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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this research project was to develop conditions and reagents required for effective targeting of the alpha-emitting radionuclide At-211 to metastatic prostate cancer cells. In the studies conducted, three different approaches to "pretargeting" At-211 to human prostate cancer xenografts were evaluated in athymic mice. One of the important questions asked was whether At-211 could be stably attached to a carrier molecule. The results obtained indicate that stable attachments can be obtained for certain types of compounds (i.e. *nido*-carboranes) but not for others (i.e. arylastatides). Effective tumor targeting was obtained in the studies, but higher than anticipated concentrations of reagents were found in non-target tissues. This finding may be due to the use of intact antibodies (Fc mediated binding). Another problem that became apparent was the fact that large quantities of biotin binding reagents (streptavidin or antibody-streptavidin conjugates) had to be used to offset the endogenous biotin released from body stores. The studies have help to delineate the requirements for pretargeting of At-211, but additional studies are required to optimize this approach.

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A. INTRODUCTION

This Final Report describes the research efforts conducted during the two years and six months funding period (July 15, 1998 thru Feb. 14, 2001) of grant number DAMD17-98-1-8500. The research project was directed at developing a new approach to the therapy of metastatic prostate cancer. The goal of the studies was to develop conditions to effectively target metastatic prostate cancer with an alpha-emitting radionuclide, astatine-211, using an antibody-based targeting system which is termed "pretargeting". To accomplish that goal, five Technical Objectives were to be addressed. The Objectives were:

Technical Objective 1: To optimize conditions for "2-step" pretargeting.
Technical Objective 2: To optimize conditions for "3-step" pretargeting.
Technical Objective 3: To evaluate the localization of At-211 labeled biotin and streptavidin using "2-step" and "3-step" pretargeting protocols.
Technical Objective 4: To evaluate the localization of At-211 and I-131 in the tibia, and evaluate the effect on PSA levels.
Technical Objective 5: To assess the dose delivered to tumor xenografts and normal tissues by I-131 and At-211 in the pretargeting studies.

The research conducted primarily addressed Technical Objectives 1, 2 and 3. The reason for the lack of completing all of the Technical Objectives is two-fold. First, an aggressive work schedule was put forth that assumed no difficulties in achieving the goals set out. As with any research, additional studies were required to attempt to circumvent unexpected results. Unanticipated difficulties with high concentrations of radiolabeled streptavidin in spleen, lung and liver, perhaps due to the use of intact biotinylated antibodies, resulted in additional studies to optimize the "2-step" pretargeting approach. In our previous experience with biotinylated Fab´ targeting we did not observe this problem [1]. Similarly, results obtained in the optimization of the pretargeting approaches suggested that there was more of a problem with endogenous biotin than had been previously believed. It was not know until a recent publication [2] that successful targeting using an antibody-streptavidin conjugate and a radiolabeled biotin derivative required large quantities (i.e. 400 μg) of antibody-streptavidin conjugate. Thus, the quantities of reagents used in many of the animal studies were insufficient to compensate for the large quantities of endogenous biotin that is apparently released from storage in the liver. It is now estimated that much larger quantities (>300 mg) of monoclonal antibody (mAb) and (>100 mg) of streptavidin (SAv) will be required to conduct the appropriate studies. We have just recently obtained these quantities of reagents for continuing studies. Further, the blood clearing reagents described in the literature, and previously used by us, were found to be non- optimal so additional studies had to be conducted in that area.

Second, the upgrade of our cyclotron to produce At-211 has gone much slower than anticipated. It has been difficult to obtain technical help on upgrading the cyclotron. Once help was obtained, it was found that the original plan for upgrading would not work, so alternative efforts have been (are being) investigated. We now produce quantities of At-211 adequate for this investigation, but this has been only a relatively recent outcome, so studies involving At-211 have been hampered.

Despite the problems encountered, we have conducted many studies and learned a great deal during the grant funding period. In our optimization studies, seventeen animal biodistributions were performed. It is important to note, the results obtained from this funding have been more extensive than might have been obtained, largely due to the fact that several new reagents were available for investigation from studies conducted under a DOE funded grant. A description of the progress made and results obtained is provided in the following section. Areas where additional studies from those first proposed were conducted are pointed out.

B. RESEARCH RESULTS

The results obtained from the research effort are outlined below in sections based on the Technical Objectives. The Tasks that were addressed are included under each Objective.

Technical Objective 1: To optimize conditions for "2-step" pretargeting.

The initial "2-step" protocol investigated herein (from proposed studies) has as a first step injection of a biotinylated monoclonal antibody (mAb) for localization of biotin on cancer cells. After a period of time for tumor localization of the biotinylated mAb, a biotin-binding clearing agent (usually avidin) is injected. This reagent does not affect the tumor-bound antibody, but rapidly clears the excess biotinylated mAb from blood. After the blood is cleared of biotinylated antibody, a second step is to inject a radiolabeled streptavidin (SAv) to bind with the biotinylated antibody on the tumor cells. Optimization of this protocol involves determining quantities and times required to gain a maximum dose on cancer cells and minimum dose in non-target tissues. The studies conducted to optimize this protocol are described below.

Task 1: Biotinylation and radioiodination of 107-1A4

Completion of this task required that we: (1) Evaluate number of biotins on mAbs when various quantities of biotinylation reagent were used in the conjugation reaction, (2) Evaluate the radioiodination of biotinylated mAbs, and (3) Evaluate the immunoreactivity and binding with streptavidin (SAv) of the radioiodinated and biotinylated antibodies.

Antibody/Antigen Characterization

At the onset of this research, the prostate specific antibody, 107-1A4 developed in Dr. Vessella's laboratory had not been completely characterized. Since that time they have published an article on its characterization [3]. Even in that paper, the antigen which the monoclonal antibody 107-1A4 binds with on prostate cells had not been characterized. Recently, it was determined that the antigen is PSMA.

Biotinylation and radioiodination 107-1A4 was the first task to be completed. Although we have developed a number of new biotinylation reagents (some with biotinidase stability), we chose to use commercially available biotin-LC NHS in these studies rather than a biotinylation reagent that we had developed because our studies indicated that there was no significant difference between them [4]. We decided that an optimal biotin loading for the initial studies would be 2-3 biotins/mAb so that there was a minimal chance of affecting the immunoreactivity or forming aggregates. Our studies indicated that when 18 equivalents of biotin-LC NHS was added, 4.5 biotins were conjugated, and when 8 equivalents of biotin-LC NHS were used, 2.4 biotins were

conjugated. A large batch conjugation of biotin-LC NHS with 107-1A4 was conducted so that the same reagent could be used in most of the animal studies conducted (except experiment where the affect of number of biotins on antibody was being evaluated).

Cell binding to Saturation Study **(Added – not originally defined as a Task)**
It was important to determine how many antigens are present on each cell so that we could estimate the maximum amount of antibody that we could bind to a cell. Thus, a cell saturation study was conducted with radiolabeled 107-1A4. In the experiment, increasing quantities of radioiodinated antibody were added to 10^6 LNCaP cells and the binding was assessed. A control antibody, MOPC-21, which does not bind the cells was employed to determine non-specific binding. It was determined that there are 1-2 x 10^6 antigens per cell. This number allows sufficient loading to permit adequate numbers of astatine-211 to conduct the therapy studies.

***Task 2*: *In vivo* evaluation of biotinylated 107-1A4**

Completion of this task required that we: (1) Prepare large enough quantities of radioiodinated and biotinylated antibodies for animal studies and (2) Conduct biodistributions in athymic mice bearing LNCaP tumor xenografts.

Animal Experiment 1 In the first animal experiment two questions were addressed. Those questions (1) How does the tumor localization of biotinylated 107-1A4 compare to that of non-biotinylated 107-1A4? and (2) How does the quantity of 107-1A4 administered affect the tumor localization? To answer the first question, equal quantities of biotinylated 107-1A4 (labeled with ^{125}I) and non-biotinylated 107-1A4 (labeled with ^{131}I) were co-injected into the mice. To answer the second question, three groups of mice were injected with different total quantities of antibody (20, 50, and 100 μg). Within each group of mice, the biodistributions were evaluated at three sacrifice times. A total of 45 mice were used. The results of the experiment are shown in Figures 1a, 1b, and 1c.

Figure 1a

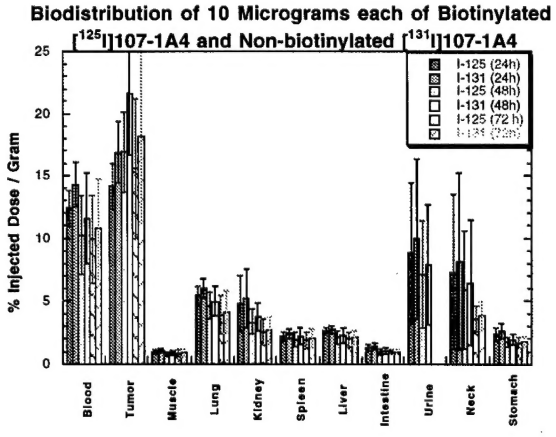
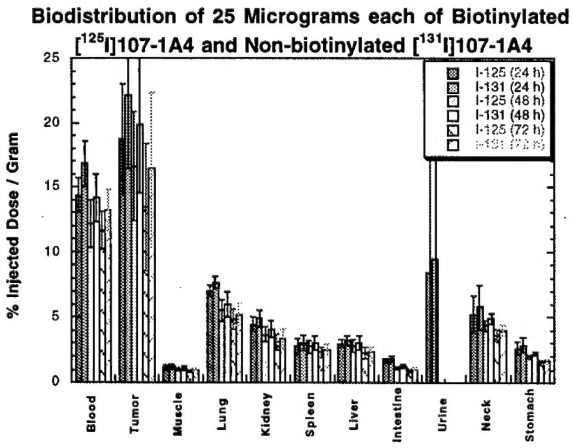
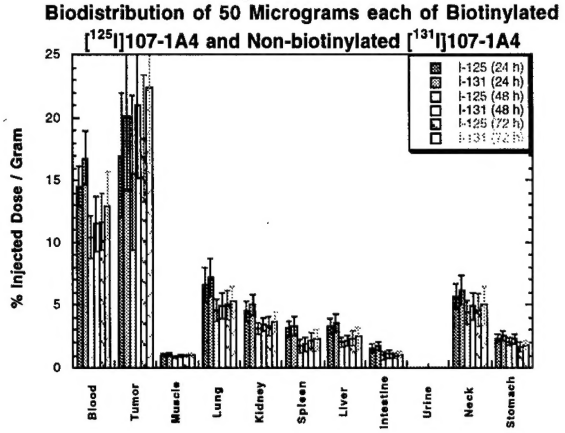


Figure 1b



It is apparent from the graphs in Figures 1a, 1b, and 1c that biotinylated 107-1A4 has a slight decrease in tumor localization (at all protein levels), but that decrease is not large enough to be a concern. Good tumor targeting and retention was obtained with all of the three quantities of 107-1A4. It was interesting to note that very similar tumor uptake was observed for even the highest quantity of antibody, suggesting that saturation had not been reached. Due to the cost of

Figure 1c:



antibody in the studies it was decided from this study that a reasonable quantity to use in subsequent experiments was 50 μg per animal.

***Task 3*: Optimize avidin as a clearing agent in 2-step pretargeting**

Completion of this task required that we: (1) Prepare biotinylated 107-1A4 and radioiodinate it [^{125}I]107-1A4, (2) Prepare [^{131}I]PIB-avidin, and (3) Conduct animal biodistribution studies, evaluating 3 quantities of avidin at 3 sacrifice times. Labeling of avidin had to be conducted by conjugation of *para*-[^{131}I]iodobenzoate [5] as direct iodination gives poor yields due to a lack of tyrosine residues on the surface.

Animal Experiment 2 The second animal experiment was designed to optimize the amount of clearing agent, avidin, used in the two step pretargeting protocol when streptavidin is the carrier of the radionuclide. In the experiment, biotinylated [^{125}I]107-1A4 was injected and 48h later, [^{131}I]PIB-labeled avidin was injected. The blood clearance and tissue concentrations of biotinylated [^{125}I]107-1A4 were evaluated in athymic mice bearing LNCaP xenografts. Varying quantities (25, 50, 100 μg) of avidin were injected and biodistributions were evaluated at 1, 2, and 4 hours post that injection.

The results of the experiment are shown in Figures 2a, 2b, and 2c. It is clear from blood levels at 72 h post injection of the antibody that the clearing agent decreased the blood concentration 40-50% (see Figure 2b for comparison). With the exception of 4h time point, the variation at the different times after injection of avidin does not show an appreciable difference. At 4h the blood concentration appears to be higher than at the earlier times, but this might be an artifact of metabolism. As expected, the radiolabeled avidin localized in kidney, spleen, and liver. Good tumor localization of the antibody was obtained, however, the concentration at the tumor appeared to decrease with time. This might suggest that the antibody is coming off of the tumor after avidin is attached. Due to this last finding, we chose to use the smallest quantity (i.e. 25 μg) of avidin in the subsequent experiments.

Note: The goal for clearing blood of biotinylated antibody was not attained. The lack of clearing blood of the biotinylated mAb may indicate that some of the mAb did not have accessible biotin for combining with avidin (solution – higher levels of biotin substitution) or it

may indicate that the avidin was not efficient at clearing. This latter point will be addressed in continued studies by adding galactose residues to avidin for more efficient clearance.

Figure 2a

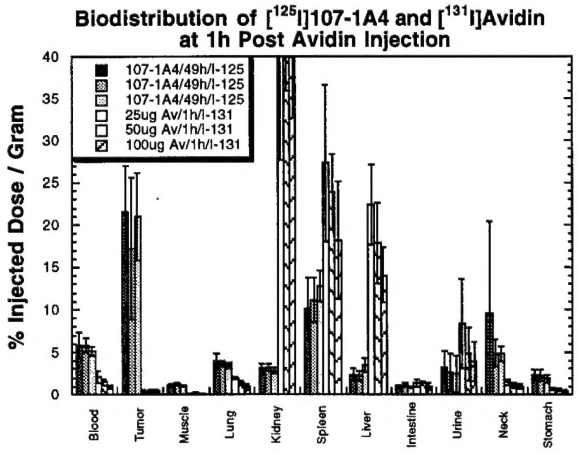


Figure 2b

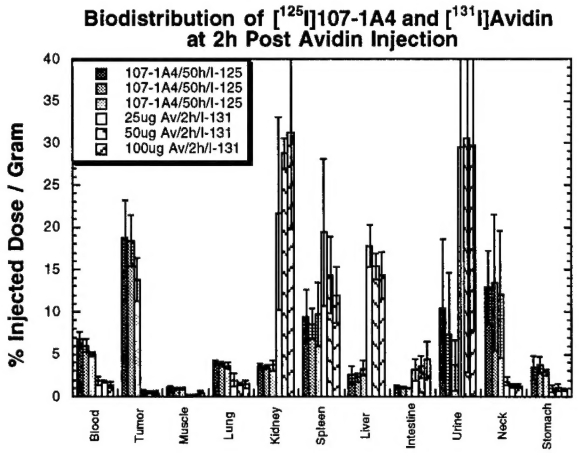
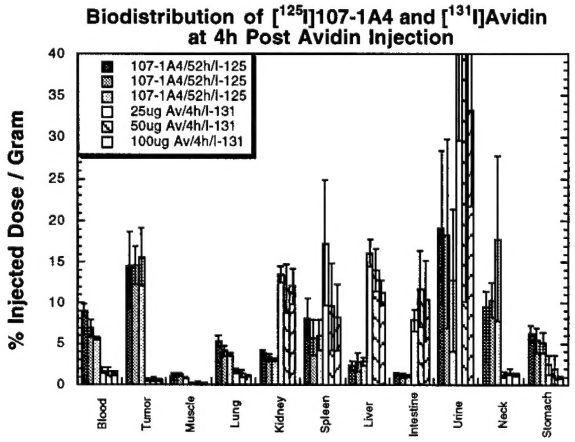


Figure 2c



***Task 4*: Optimize radiolabeled streptavidin in 2-step pretargeting**

Completion of this task required that we: (1) Prepare radioiodinated streptavidin, (2) Demonstrate that succinylated streptavidin can be radioiodinated, (3) Characterize the radioiodinated and succinylated SAv by HPLC, SDS-PAGE, IEF, (4) Prepare [^{125}I]107-1A4 and [^{131}I]PIB-succinylated streptavidin, and (5) Conduct animal studies, evaluating 3 quantities of streptavidin at 4 sacrifice times.

Animal Experiment 3 The third animal experiment was designed to evaluate the quantity and time for injection of radiolabeled streptavidin to obtain optimal tumor co-localization of biotinylated [^{125}I]107-1A4 and succinylated [^{131}I]streptavidin in LNCaP xenografts in athymic mice when avidin is used as a clearing agent. In the experiment, varying quantities of succinylated streptavidin were evaluated at four times (1,3,7, and 24 h pi streptavidin) to determine what gave optimal tumor localization and biodistribution.

The results of the experiment are shown in Figures 3a, 3b, 3c, 3d, 3e, 3f, 3g, and 3h. Because of the large amount of data, the distribution of [^{125}I]107-1A4 and succinylated [^{131}I]streptavidin have been put on separate graphs. Thus, graphs 3a/3b, 3c/3d, and 3e/3f are pairs of the same sets of animals that have had these reagents co-injected. Graphs 3g and 3h compare the data from the experiments in a different manner to show effects. Examination of the graphs for the antibody (3a,3c,3e) shows that at time of sacrifice, the tumor-to-blood ratios of 2-3:1 were obtained. It is interesting to note that the blood levels appear to go up with time, perhaps indicating that metabolism or release of radiolabeled streptavidin is occurring. In the antibody distributions, fairly high spleen and liver concentrations are seen with all of the quantities of streptavidin used, but the quantity decreases significantly over the 24 h period.

At the initiation of this experiment, it was thought that streptavidin might localize fairly readily to the tumor xenografts, however, it is very clear from graphs 3b,3d,3 f that is not the case. Indeed, not until 24 h pi streptavidin does it localize to the maximum quantity in the tumors, and at that time the tumor-to-blood ratios are similar to those obtained for the intact antibody. It is

Figure 3a

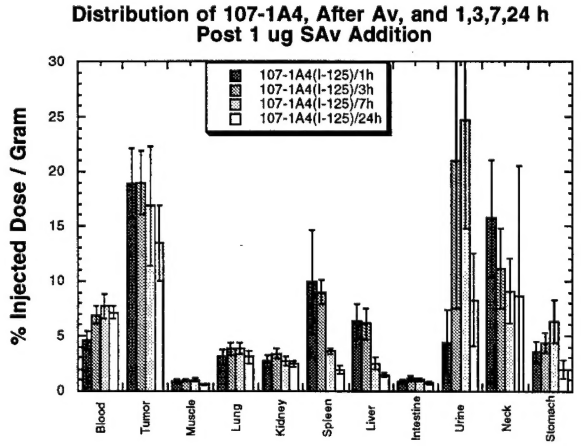


Figure 3b

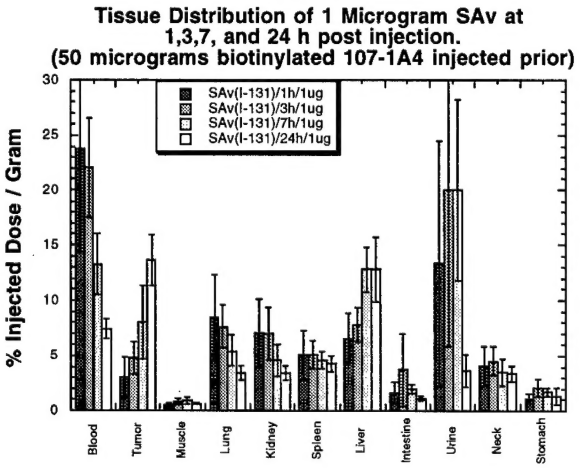


Figure 3c

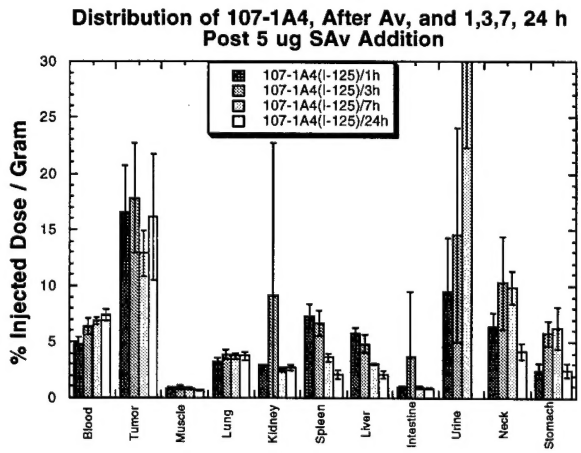


Figure 3d

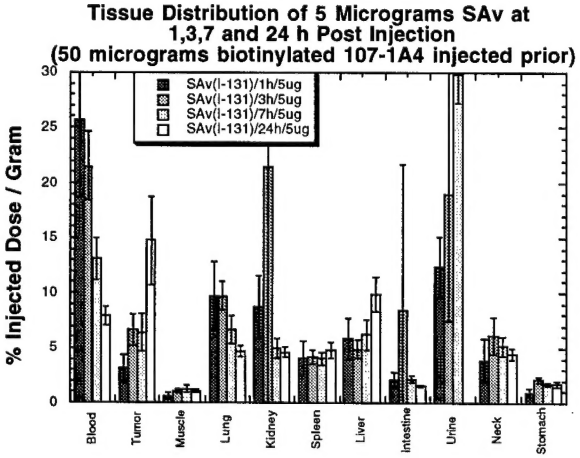


Figure 3e

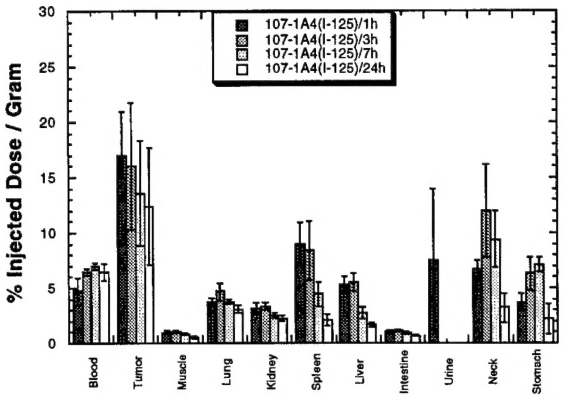
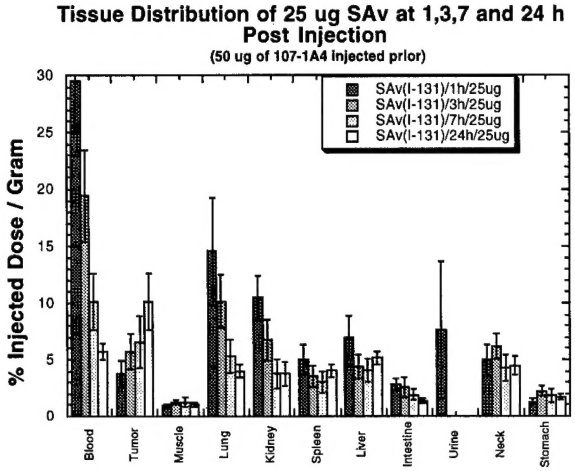


Figure 3f



thought that this slow binding with biotinylated 107-1A4 at the tumor may be due to the highly necrotic nature of this xenograft. Another observation that can be made is that at the lower quantities of streptavidin evaluated (1 and 5 μg) the liver concentration increases with time. Thus, in our optimized studies, the minimum time after injection of radiolabeled streptavidin (for solid tumors) is 24h and the quantity used is greater than 5 μg.

To obtain a better comparison of the blood and tumor concentrations of radiolabeled streptavidin at the various times and quantities of streptavidin injected, a graph was made. That graph is shown in Figure 3g. It is very apparent from the graph that the 24 h period is best, but it suggests that there may be a problem with a short half-lived radionuclide (At-211) in solid tumor targeting with this agent. The fact that the % injected dose / gram quantity appears to decrease when the higher concentration of streptavidin was added was a bit troubling until it was assessed as pmol/g relative to the biotinylated antibody (graph 3h). Importantly, at the highest quantity of streptavidin (25 μg), a 1:1 ratio was obtained for streptavidin-to-antibody at the tumor.

Figure 3g

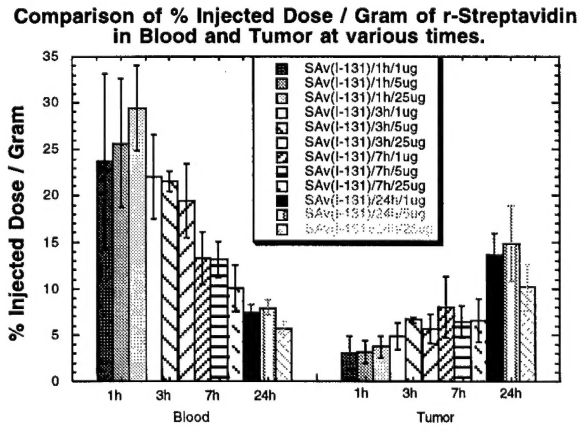
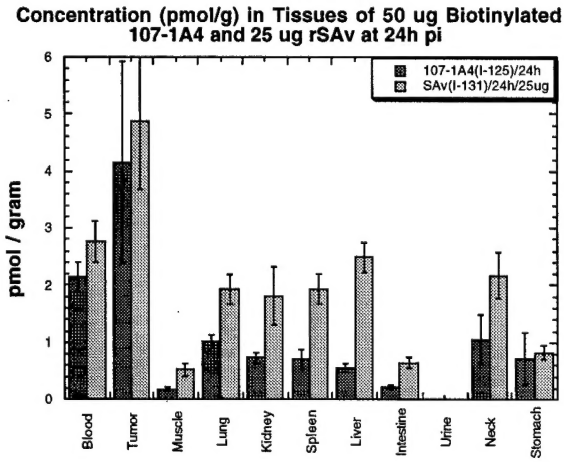


Figure 3h



Animal Experiment 4 The fourth animal study was designed to evaluate the co-localization and tumor targeting of radiolabeled streptavidin with the antibody 107-1A4 which had varying quantities of biotin conjugated. In the experiment, 50 µg of biotinylated [^{125}I]107-1A4 with 3 different conjugation levels of biotin (2.3, 4.5, and 6.8 biotins/mAb) was injected into 3 sets of 15 athymic mice bearing LNCaP xenografts (45 mice total) . After 48 h, 25 µg of avidin was injected into each mouse. After 1 h, 20 µg of [^{131}I]streptavidin was injected and animals were sacrificed at 24h, 48h, and 72 h post injection.

The results are shown in Figures 4a,4b,4c,4d,4e,and 4f. Because of the large amount of data, the distribution of biotinylated [^{125}I]107-1A4 and succinylated [^{131}I]streptavidin have been put on separate graphs. Thus, graphs 4a/4b, 4c/4d, and 4e/4f are pairs of the same sets of animals that have had these reagents co-injected. Each graph contains a comparison of localization at the various biotin conjugation levels. The different sets of graphs depict the results based on the time of sacrifice.

Figure 4a

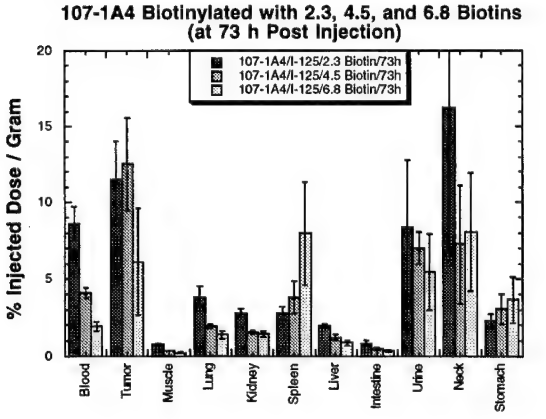


Figure 4b

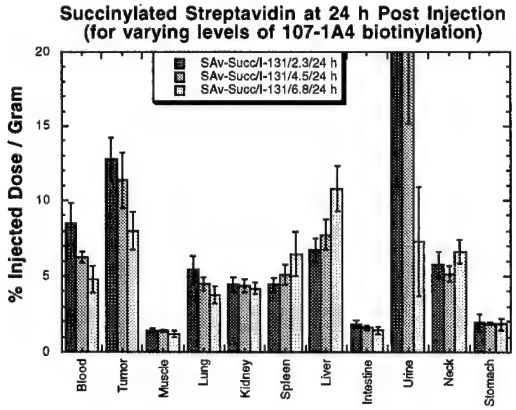


Figure 4c

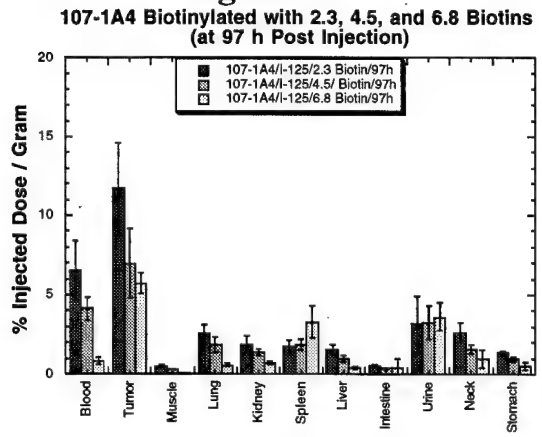


Figure 4d

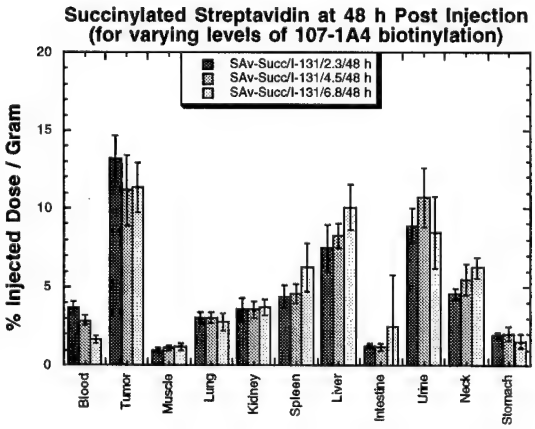


Figure 4e

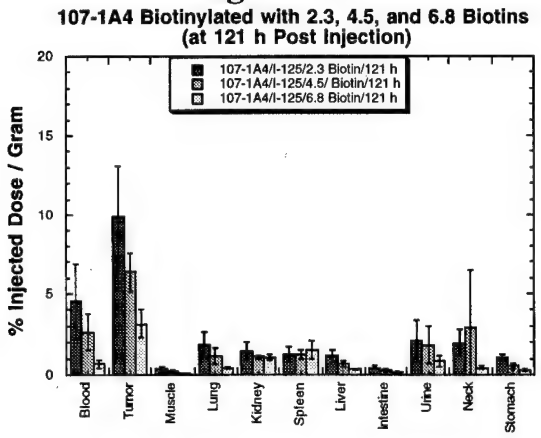
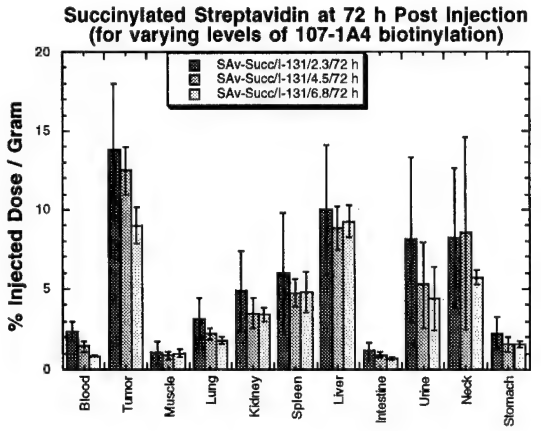


Figure 4f



It is apparent from the 24 h time point (4a) that as the amount of biotin conjugated to the 107-1A4 increases, the concentration in blood and tumor decreases significantly, and the concentration in the spleen increases. With time (4c: 48h; 4e: 72h) the antibody concentration falls as would be expected, but the more rapid decrease in the blood and tumor for higher levels of biotinylation is still apparent. In contrast to this, the differences seen in blood and tumor concentrations for radiolabeled streptavidin is not as large as those seen for the biotinylated antibody. Indeed, the quantities of radioactivity are significantly higher for the streptavidin in the tumor than is measured for the biotinylated antibody. This fact may be an indicator that the antibody-streptavidin complex is internalized and the antibody is degraded much more quickly than streptavidin. It is known that streptavidin is very slowly metabolized. The other major observation about the distribution of succinylated streptavidin is that the spleen and liver are quite high, particularly in contrast to the antibody. This again may be a sign of slow degradation of the streptavidin if the antigen/antibody/streptavidin complex comes is shed and the all are broken down except for streptavidin.

The results of this experiment suggest that in this tumor xenograft, the antigen that is bound is shed and internalized. It has been shown recently that the antigen for 107-1A4 is PSMA, which is shed and internalized. Thus, the localization in spleen and liver may be a function of the antibody and its target antigen. To evaluate this possibility, an animal study was conducted with another antibody (experiment 5, below). Alternatively, since the amount of streptavidin in spleen and liver is much higher than the antibody, we reasoned that there may be something about the succinylated streptavidin that caused it to go to or be retained in these tissues. An animal experiment (experiment 6) was conducted to examine the latter case.

Animal Experiment 5 **(Added – based on results described above)** In the fifth animal experiment, we investigated the issue of whether the biotinylated antibody directed the localization of radiolabeled succinylated SAv in non-target tissues by conducting the same study (or quite similar) using the renal cell carcinoma antibody, A6H, targeted against a renal cell carcinoma (RCC) xenograft, TK-82. It has been established that the antigen is not shed or internalized in the RCC xenograft. The biodistributions study was conducted exactly as described for experiment 4 (above), except biotinylated A6H was used in the place of biotinylated 107-1A4. Results of the biodistribution are provided in Figures 5a, 5b, 5c, 5d, 5e, and 5f. Again the results are paired with regards to mAb and SAv, and according to the number

Figure 5a

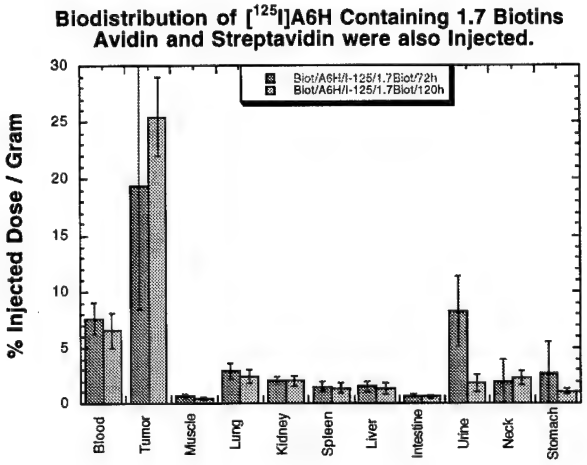


Figure 5b

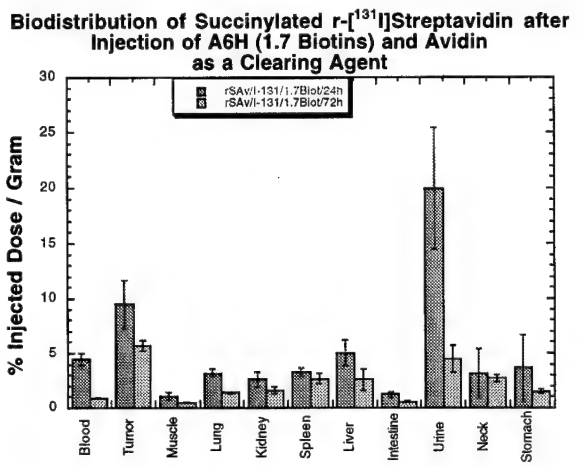


Figure 5c

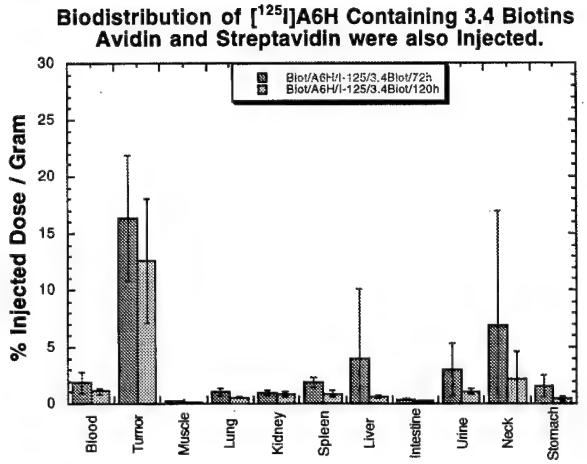


Figure 5d

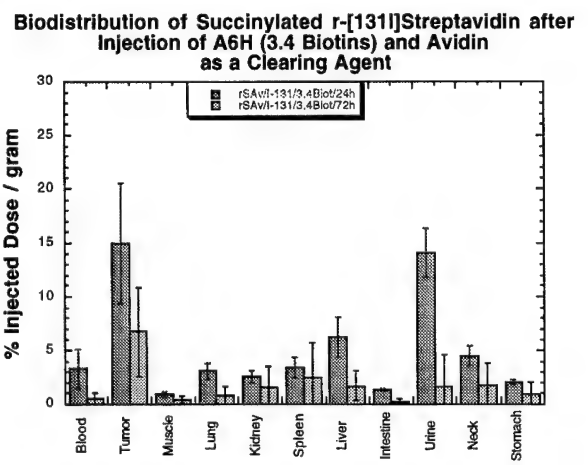


Figure 5e

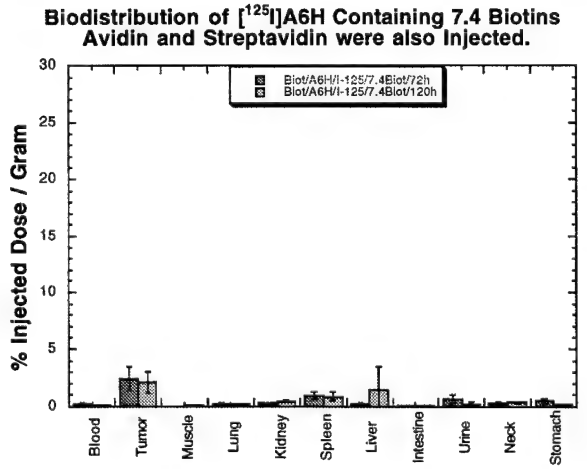
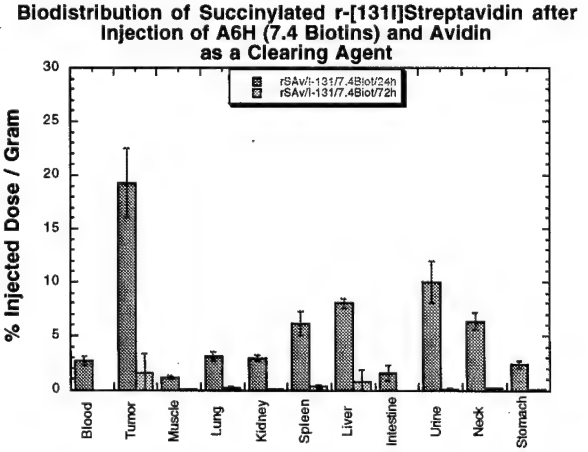


Figure 5f



of biotins / mAb 1.7 for 5a/5b; 3.4 for 5c/5d; and 7.4 for 5e/5f. The radiolabeled SAv graphs (i.e. 5b, 5d, and 5f) are most important as they represent the distribution of the therapeutic radionuclide that one would expect in the experiments. It is clear that the antibodies (107-1A4 and A6H) give similar results. However, the A6H results look worse than the 107-1A4 results. Despite these results we felt that it was important to conduct an experiment where we looked at the distribution of radiolabeled succinylated streptavidin over 4 day time period. That experiment and the results obtained are described below.

Animal Experiment 6. A sixth experiment was conducted wherein the 107-1A4 was biotinylated to have the intermediate quantity of biotins/mAb (i.e. 3-4 biotin/mAb) and its biodistribution relative to succinylated SAv was determined at three time points. In the experiment, 100 µg of biotinylated [^{125}I]107-1A4 was injected into mice and after 48 h, 50 µg of avidin was administered. After 1 h post the avidin injection, 100 µg of succinylated [^{131}I]SAv was injected and the distributions were examined at 24h, 48h, and 96h post the succinylated [^{131}I]SAv injection. The results are shown in Figures 6a and 6b. The tumor localization of [^{131}I]SAv is reasonable (13-20%ID/g) and stable with time. Importantly, the blood concentrations decrease with time and good tumor-to-blood ratios are obtained. However, the concentrations of labeled SAv in lung, kidney, spleen and liver are very troublesome.

Figure 6a

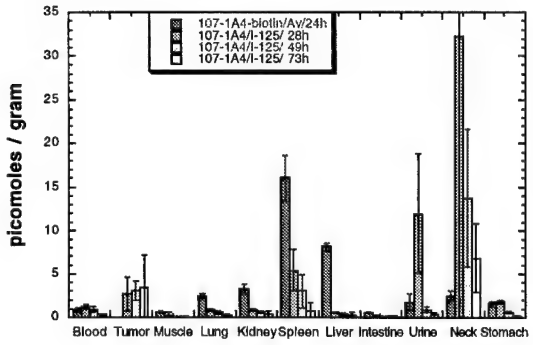
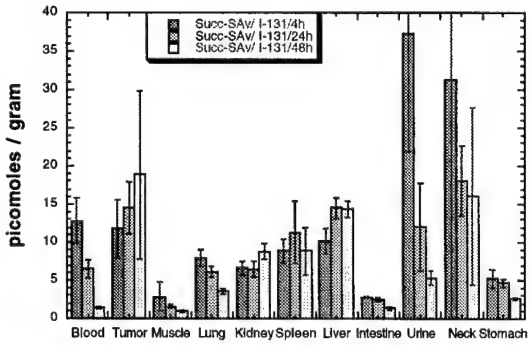


Figure 6b



At this point it does not appear the "2-step" approach is viable due to the high concentrations found in non-target tissues. Additional studies need to be conducted to determine if the non-target tissue localization (other than neck and stomach) is due to a property of the intact mAb. Good tumor localization is obtained within 24h with the targeted SAv, but the concentration of radionuclide in non-target tissues is too high for this to be an effective delivery method. Continuing (separately funded) studies will evaluate the use of mAb Fab´ fragments for targeting.

Technical Objective 2: To optimize conditions for "3-step" pretargeting.

The "3-step" protocol investigated herein is quite similar to the "2-step" protocol in that it has as a first step injection of the biotinylated monoclonal antibody and the second step (after blood clearance) injection of succinylated streptavidin. The important difference is that the streptavidin does not carry the therapeutic radionuclide, rather, in a third step (after a second blood clearance step), the radionuclide is administered on a biotin derivative. Because of the

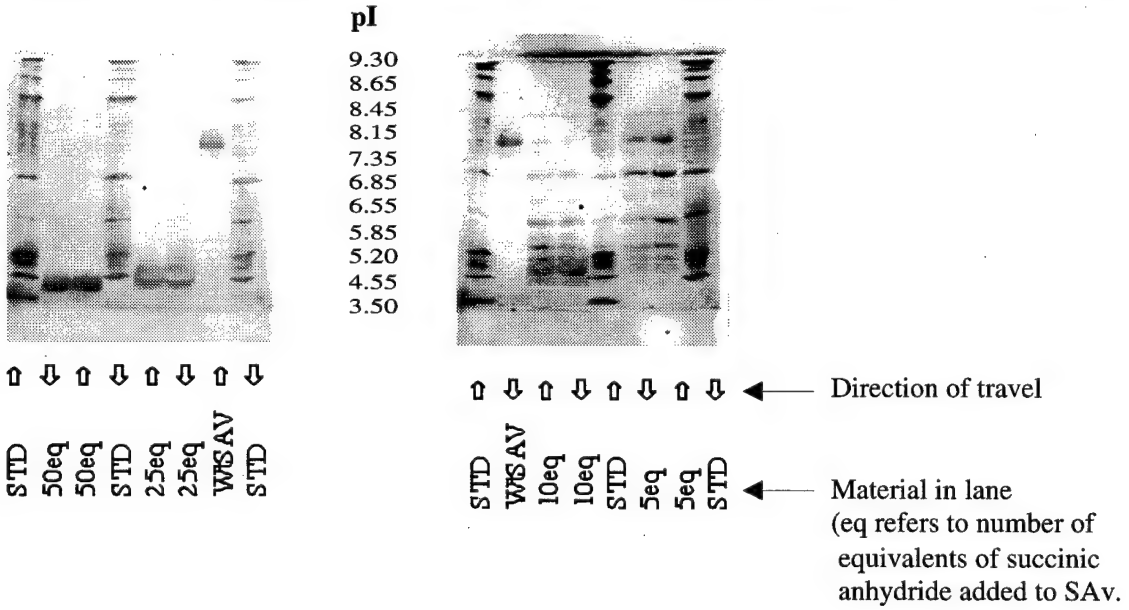
earlier studies, optimization of this protocol involves optimizing the properties, quantities and timing of succinylated streptavidin, biotinylated clearing agent, and biotin derivative to be used.

Task 5: Optimize succinylated streptavidin in 3-step pretargeting

Completion of this task required that we: (1) Prepare biotinylated 107-1A4, (2) Prepare succinylated streptavidin and characterize it by HPLC, SDS-PAGE, IEF, (3) Radioiodinate biotinylated [^{125}I]107-1A4 and succinylated [^{131}I]streptavidin, and (4) Conduct animal studies, (a) evaluating quantities and timing of administering succinylated streptavidin,(b) evaluating the biodistribution of radioiodinated biotin derivatives, and (c) evaluating the timing and quantities of administered reagents.

Succinylated Streptavidin Our previous studies demonstrated that succinylation of streptavidin decreased the kidney accumulation significantly [6]. During the studies conducted, a question of whether succinylated streptavidin was localizing (by itself) to the RES system arose. Since we had not previously studied how varying amounts of succinylation affected streptavidin's biodistribution, we felt that it was important to add that experiment to the proposed studies. Varying quantities of succinylation of streptavidin was obtained by reaction with 5, 10, 25 or 50 equivalents of succinic anhydride. Our previous studies had all been carried out by reaction of 50 equivalents of succinic anhydride. The IEF gels (shown below) clearly show the different levels of succinylation (and pI ranges of succinylated streptavidin) for the varying quantities of succinic anhydride offered. This material was studied in animal experiment 7 (see next section).

Figure 1: IEF gels of streptavidin after various levels of succinylation



Animal Experiment 7 **(Added - based on results of other studies)**
The seventh animal experiment was designed to evaluate the effect of various levels of succinylation on the in vivo distribution of streptavidin. This had not been evaluated in our previous publication on succinylated streptavidin [Wilbur, 1998 #10408]. In the experiment, streptavidin which had been reacted with 5, 10, 25 or 50 equivalents of succinic anhydride were

radiolabeled (4 preps) with ^{125}I. A control that contained radiolabeled [^{131}I]streptavidin was also made for co-injection. A total of 20 mice were co-injected with succinylated [^{125}I]streptavidin and [^{131}I]streptavidin and biodistributions were obtained at 24 h post injection.

The results for the succinylated streptavidin by itself are shown in Figure 7a, and a comparison of the lowest and highest succinylation levels compared with unmodified streptavidin are shown in Figure 7b (unaltered streptavidin). The data indicate that the level of succinylation dramatically influences the kidney concentration of streptavidin (see Fig. 7a). The concentration of succinylated and unmodified streptavidin are very similar in all other tissues (see Fig. 7b).

Figure 7a

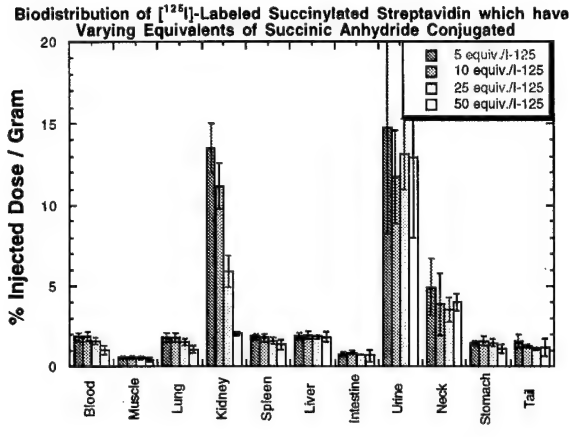
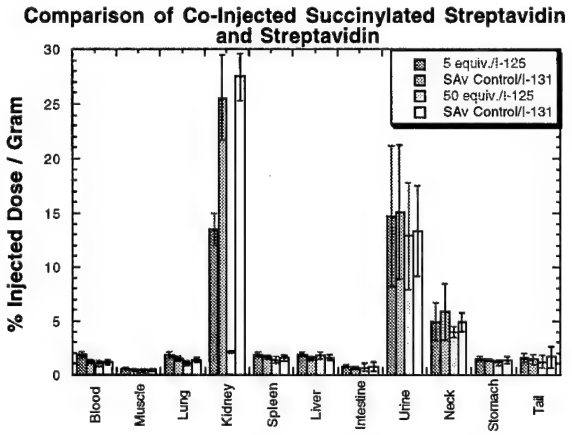


Figure 7b



The results obtained in this experiment indicate that the properties of succinylated streptavidin has little or nothing to do with the high liver and spleen concentrations observed in experiment 4. Therefore, a logical explanation is that succinylated streptavidin was carried to those tissues as complexes with antibody or antigen/antibody complexes.

Task 6: Optimize asialoorosomucoid as clearing agent in 3-step pretargeting

At the onset of the proposed studies, the reagent of choice for clearing biotin binding proteins from blood appeared to be biotinylated asialoorosomucoid protein. Thus, we used this reagent in our studies. To complete this task we had to: (1) Prepare asialoorosomucoid by treatment with neuraminidase, (2) Biotinylate asialoorosomucoid (BAOM) and characterize it by HPLC, SDS-PAGE, IEF, (3) Prepare biotinylated 107-1A4 and succinylated streptavidin, (4) Radiolabel succinylated [^{125}I]streptavidin and [^{131}I]BAOM, and (5) Conduct animal studies, evaluating 3 quantities of BAOM at 3 sacrifice times.

Animal Experiment 8 The eighth animal study was designed to optimize the quantity of clearing agent used in a three step pretargeting protocol. In that study, biotinylated 50 µg of [^{125}I]107-1A4 was injected into 15 mice. After 48 h, 25 µg of [^{131}I]streptavidin was injected into each mouse. After another 24 h, varying quantities (20, 50 or 100 µg) of biotinylated asialoorosomucoid (BAOM) were injected, and 4 h later the mice were sacrificed. The data obtained are plotted in Figures 8a ([^{125}I]107-1A4) and 8b ([^{131}I]streptavidin). The data indicate that the maximum clearance is obtained (even) with the smallest quantity of BAOM. Plotting the concentration of reagents in picomole / gram (Figure 8c) shows that the maximum amount of streptavidin was bound (1:1 ratio) to the biotinylated antibody at the tumor.

Figure 8a

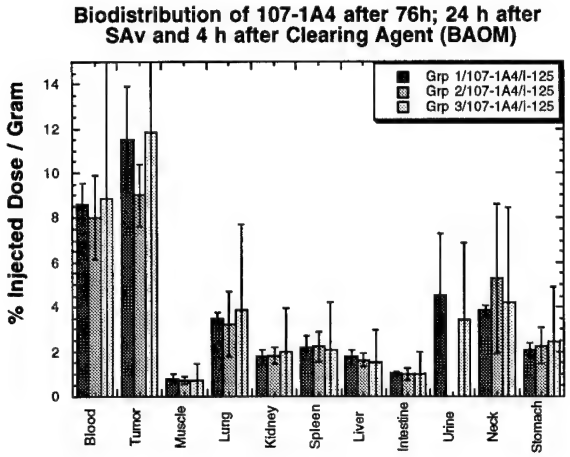


Figure 8b

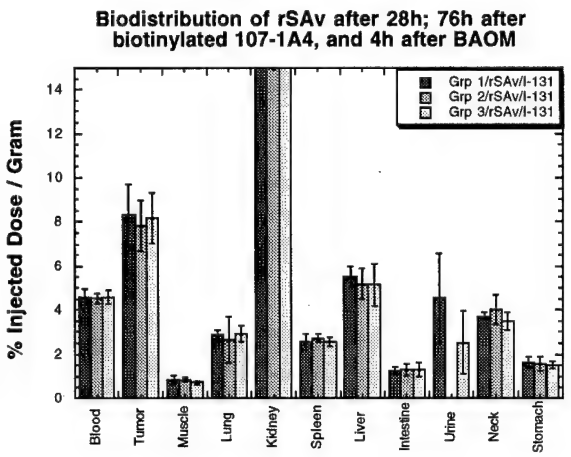
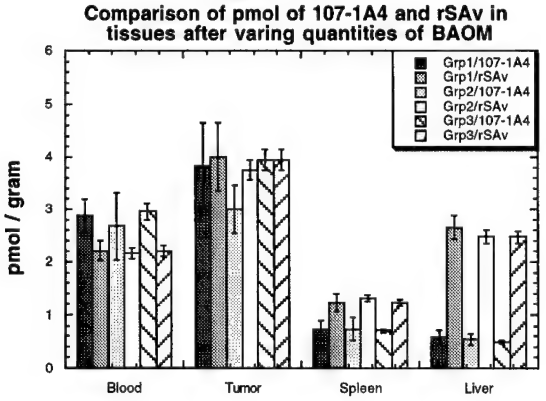


Figure 8c



Task 7: Optimize radiolabeled biotin in 3-step pretargeting (quantities and timing)

This task was not conducted as the results obtained in the foregoing experiments indicated that better (more selective) targeting was required. Additional studies were added wherein a "2-step" pretargeting protocol using mAb-SAv conjugates directly for targeting, rather than conducting two steps (biotinylated mAb, then succinylated SAv). Those studies are included later in this report.

Technical Objective 3: To evaluate the localization of At-211 labeled biotin and streptavidin using "2-step" and "3-step" pretargeting protocols.

A very important aspect of the proposed studies was the production of At-211 and its use in labeling of the carrier molecules. Some of the studies were conducted in parallel with the foregoing studies, so we did not have the benefit of the results to aid our research direction. However, all of the studies conducted helped us understand the experimental parameters required to label molecules with At. In this technical objective, it was important to first demonstrate that At-211 could be attached to streptavidin (2-step approach) or biotin (3-step approach) in high yield and resulted in binding that was stable to in vivo dehalogenation.

Task 8: Conduct preliminary production of At-211

At the onset of the funded proposal, we were planning to upgrade the cyclotron to produce the requisite alpha-beam for production of At-211. Dr. Reudi Risler has spent considerable time and effort at obtaining an alpha-beam and conducting irradiations for us. After many difficulties in getting a contract with a group (from TRIUMF/UBC, British Columbia, Canada) that could make design modifications to the cyclotron it was found that the original thoughts on alterations to upgrade the cyclotron were not feasible. Fortunately, Dr. Risler tried many different simple alterations and found that some combinations allowed production of small quantities of At-211. A goal of obtaining 50 μA of alpha-particle beam on the target has been set. We believe that beam intensity will permit us to prepare ample quantities of At-211 for clinical studies (i.e. 50-100 mCi at end-of-bombardment). We presently put 10-12 μA on our bismuth targets because the target assembly that we have will not permit higher beam amperage. However, Dr. Risler has been able to obtain a stable beam at around 30 μA and short runs have been made of up to 40 μA. Dr. Risler is continuing to reconfigure and redesign parts of the cyclotron to obtain the targeted 50 μA beam. We are also having a new target assembly designed and built that will accommodate the higher beam amperages.

Over the past year we have conducted 8 irradiations to produce At-211. The amount of At-211 in the target has ranged from 1.3 – 4.0 mCi in a 2 h irradiation. Using the distillation apparatus previously described [7], we have been able to obtain 60-75% of the At-211 from the targets (1.0-2.6 mCi) for use in subsequent labeling studies.

Task 9: Synthesize nido-carborane for conjugation to streptavidin

To complete this task, we had to: (1) Synthesize and characterize *nido*-carboranes for conjugation, (2) Conjugate varying quantities to streptavidin, (3) Evaluate radioiodination of *nido*-carborane streptavidin conjugates, (4) Evaluate succinylation and radioiodination of conjugated streptavidin, and (5) Characterize the products by HPLC, SDS-PAGE, IEF.

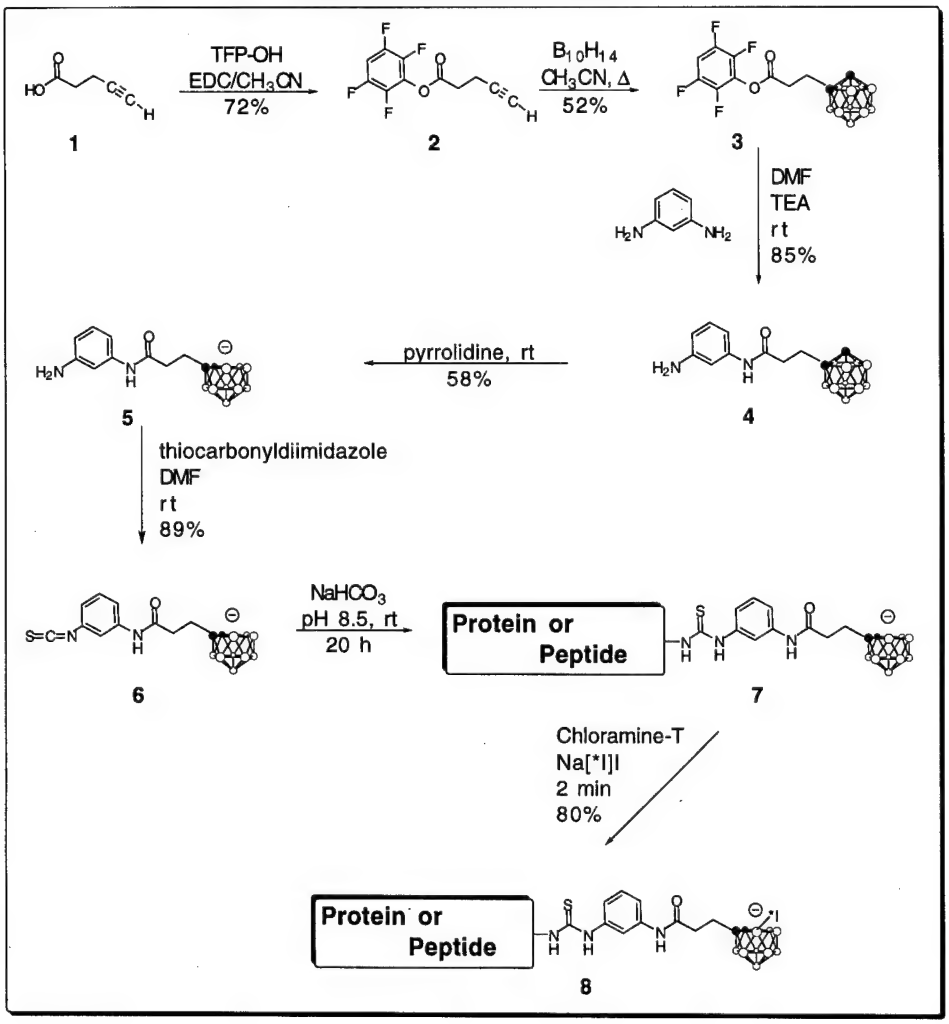
In studies prior to initiation of this research effort, we prepared several *nido*-carborane derivatives and demonstrated that they radioiodinated efficiently [Wilbur, 1994 #8900; Wilbur, 1995 #8901]. Indeed, we found that *nido*-carboranes reacted some 50x faster with radioiodine than did tyrosine. In other studies we (in collaboration with Prof. Michael Zalutsky at Duke University) demonstrated that *nido*-carboranylpropionic acid could be rapidly and efficiently astatinated [Wilbur, 1997 #10546]. Therefore, we felt that it was reasonable to consider using *nido*-carboranes that have functional groups for protein conjugation for direct introduction of radioiodine and astatine-211 into proteins. This is particularly important with astatine-211 as direct labeling results in a labile attachment of that radionuclide [Visser, 1981 #8107; Visser, 1981 #8106; Vaughan, 1978 #8041]. Although we attempted to prepare the *nido*-carborane conjugation reagent describe in our proposed studies, we were unsuccessful. However, during this grant period we did successfully prepare two new *nido*-carborane reagents, **6** and **13**, that can be conjugated with proteins (see Schemes 1 and 2).

We used avidin as a model protein to study direct labeling with radiohalogens. The rationale for this is that we routinely have to radiolabel this protein by conjugation of radioiodinated iodobenzoate N-hydroxysuccinimide ester ([*I]PIB)[Wilbur, 1989 #10488] because there are no available tyrosines to radioiodinate. The radiochemical yield for the [*I]PIB labeling procedure

is at best 60%. Thus, we have studied the conjugation of **6** with avidin and found that using more than 5 equivalents (offered) caused rapid (visible) aggregation of the protein. However, at 5 equivalents offered we obtain *nido*-carborane conjugation which was soluble, and we obtained 80-95% radioiodination yields (get 6-29% yields of partially labile radioiodine without *nido*-carborane conjugated). This demonstrates unequivocally that direct radioiodination can be accomplished on *nido*-carborane conjugates. This was the first example of direct labeling of a prosthetic group other than phenol (e.g. Wood's reagent) for protein labeling.

We have also conducted a number of conjugations of **6** with streptavidin. In contrast to the avidin reactions, streptavidin does not aggregate. We believe the propensity for avidin to aggregate is due to its high pI (10) relative to that of streptavidin (pI = 7.2). Since there are five available tyrosines on each subunit of streptavidin (20 total) we normally directly label with radioiodine. With this number of tyrosines, it is expected that some of the radioiodine will go on tyrosines even when **6** is conjugated. Indeed, we have tried to determine what percentage goes on conjugated **6** vs tyrosine residues, but that has proven to be difficult. Our studies have shown

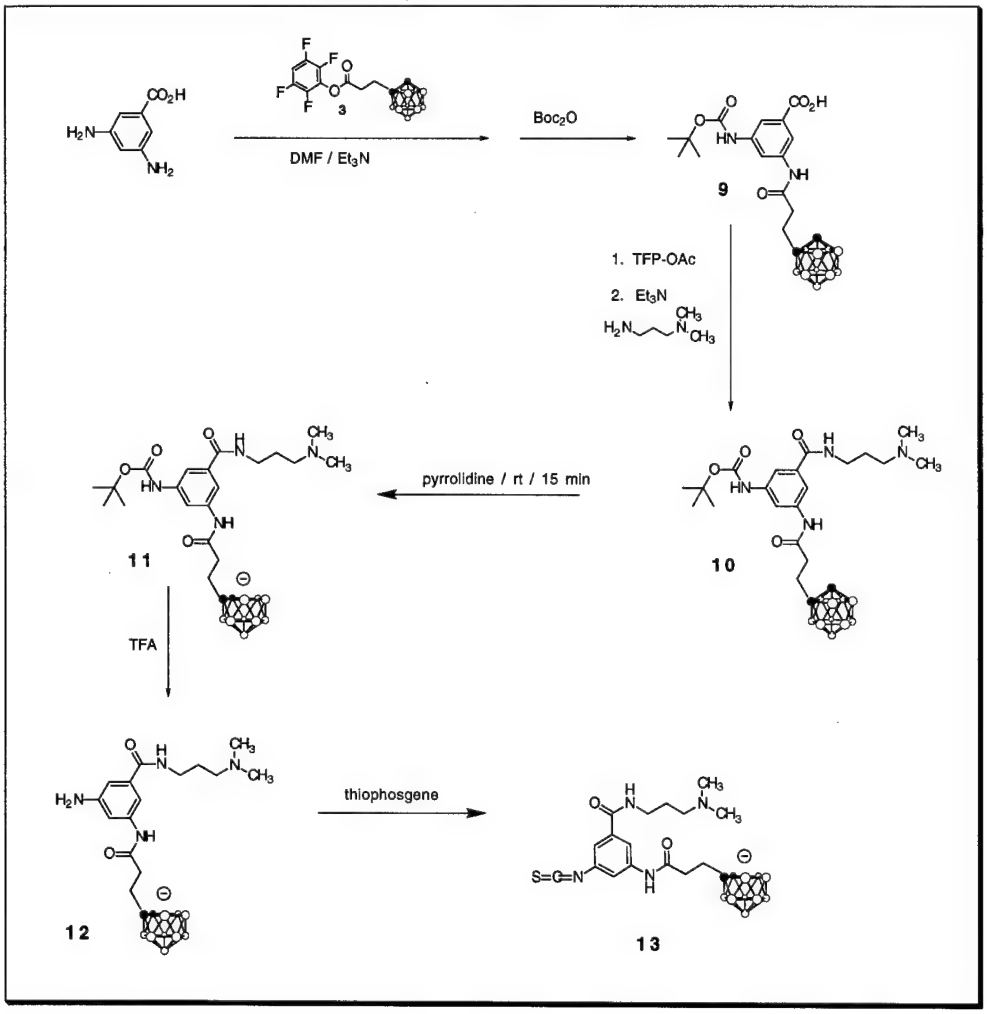
Scheme 1: Synthesis, protein conjugation and radioiodination of a *nido*-carborane derivative



that with increasing amounts of **6** offered, a decreasing pI value is obtained (by IEF analysis). Size exclusion HPLC analysis of avidin and streptavidin which has been conjugated with **6** show that as the amount of **6** offered increases, the shape and retention of the peaks obtained change. This fact suggested that we might be changing the molecules quite a lot. Therefore, we also studied binding of biotin (as a biotin-cyanocobalamin adduct) with the modified proteins. Although we do not have conclusive data at this time, it appears that conjugation of **6** also significantly alters the binding of biotin. We believe we know the reason for this and have some experiments planned that might alleviate that problem.

The problem of protein aggregation with *nido*-carborane conjugates was believed to be brought about by interaction of the *nido*-carborane with protonated amines on a second protein molecule. Based on this belief, we synthesized an alternate *nido*-carborane containing molecule, **13**, that could be conjugated with proteins. That synthesis is shown in Scheme 2. Protination of the dimethylamine in aqueous solution will provide a built-in counter-ion for the negatively charged *nido*-carborane moiety. Indeed, the propensity for avidin and (to a less extent) SAv to aggregate when being conjugated with **6**, was dramatically reduced when **13** was conjugated.

Scheme 2: Synthesis of Conjugation Moiety that Contains a *nido*-Carborane with a Dimethylamine Counter-ion



Task 10: Conduct At-211 labeling of streptavidin

In our original proposal, At-211 labeling was to be conducted with N-succinimidyl *p*-[^{211}At]benzoate ([^{211}At]PAB), succinylated after labeling. However, the results obtained with radioiodination of the *nido*-carborane conjugated SAv were very encouraging, and it was felt that this task should be modified to use the *nido*-carborane labeling method. The conjugation of *nido*-carborane **6** with SAv was readily accomplished and succinylation of the remaining amines provided SAv with a pI in the region that we felt it would not be sequestered in the kidneys. At-211 labeling was readily accomplished using chloramine-T as the oxidant. The At-211 labeled succinylated SAv was purified by size exclusion chromatography and used for in vivo evaluation.

Task 11: Evaluate in vivo distribution of [^{211}At]streptavidin

Animal Experiment 9 The biodistribution of At-211 labeled succinylated streptavidin was compared with I-125 labeled SAv in athymic mice at 1, 4, and 24 h post injection. The results obtained are shown in Figures 9a, 9b, and 9c. It is important to note that the concentrations of At-211 and I-125 in the various tissue is very nearly the same, indicating that the At-211 label is stable to in vivo dehalogenation. Unfortunately, the blood concentration of *nido*-carborane modified SAv is at least 3x higher than observed for non-modified SAv [8]. Further, it appears that the high tissue concentrations (other than blood) do not significantly decrease with time.

Figure 9a

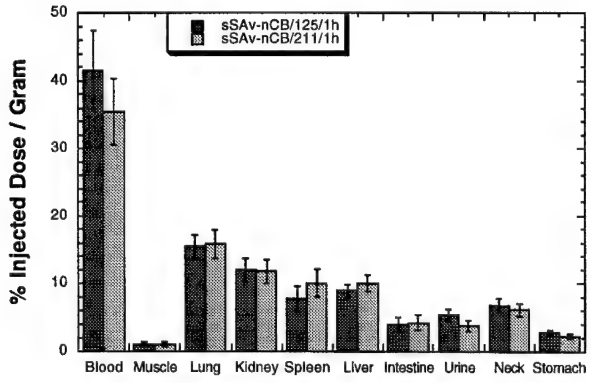


Figure 9b

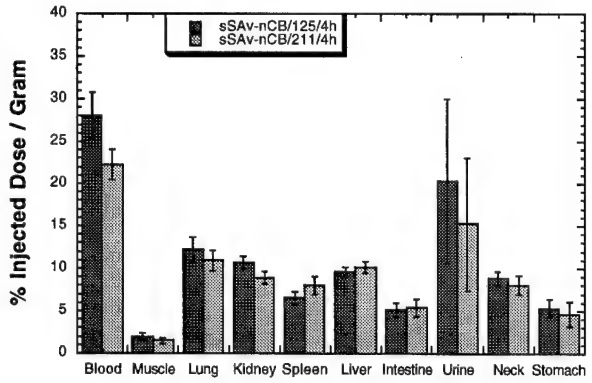
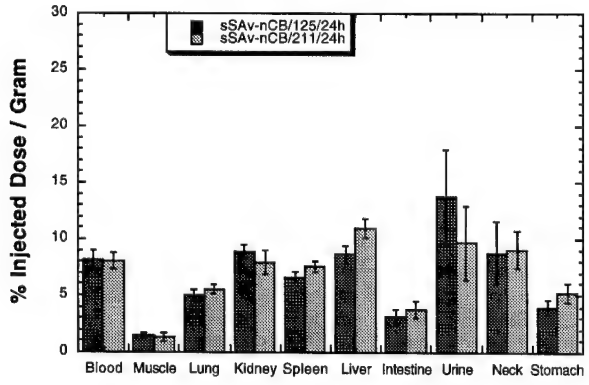


Figure 9c



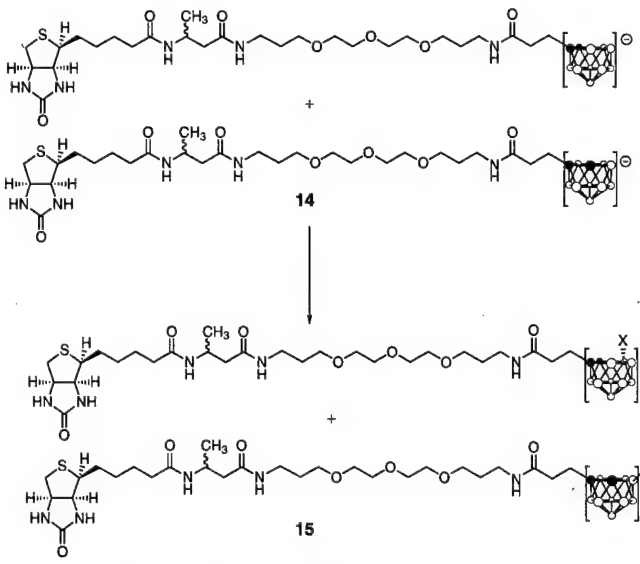
Task 12: Optimize 2-Step pretargeting of At-211

The results obtained with At-211 labeled succinylated and *nido*-carborane conjugated SAv, along with the results obtained for the 2-step pretargeting approach, strongly suggest that it is not a viable approach to cancer cell targeting for At-211. For this reason, the optimization of the 2-step approach was not conducted.

Task 13: Conduct At-211 labeling of Biotin Derivatives

As part of our studies, we have: (1) Synthesized several biotin derivatives, (2) Evaluated their At-211 labeling and (3) Conducted in vivo biodistributions to determine stability. Early in vivo studies with At-211 labeled molecules suggested that stability of the At-211 could be obtained if they were not metabolized. To circumvent this instability, we decided to prepare some biotin derivatives that contained *nido*-carboranes for labeling. We previously prepared a biotin derivative **14** (as a diastereomeric pair, see Scheme 3), astatinated it, and evaluated the distribution in mice [Wilbur, 1997 #10546]. While the results were encouraging, other studies demonstrated

Scheme 3: Astatination of a Biotin derivative containing *nido*-carborane that was previously synthesized

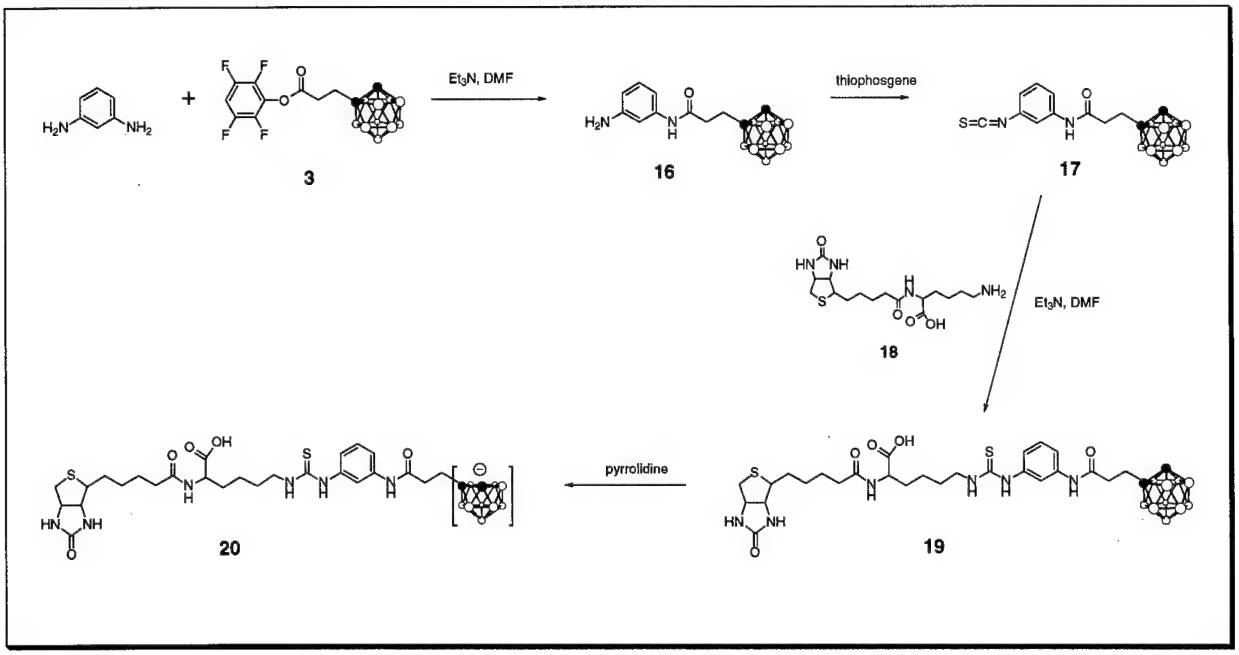


*Two additional isomers can be formed, but the isomers shown are expected to be the major products as the steric interaction of the halogen and the substituent on the borane carbon is minimized.

that the biotin derivative used is susceptible to biotinidase cleavage. Therefore, we decided that it was best to develop another *nido*-carborane containing biotin reagent that was stable to serum biotinidase [Wilbur, 1997 #10426]. We initially prepared a biotin derivative (not shown) containing a thiourea bond in the place of the biotinamide bond. It was thought that the thiourea would not be cleaved by the amidase-based biotinidase enzyme. However, after preparing this compound, we have conducted related studies which indicated that biotin moieties with a thiourea bond loose their desired high binding affinity to avidin and streptavidin [9]. We

demonstrated that conjugates of biotin and amino acids (α-amine conjugated) which result in having a carboxylate alpha to the biotinamide bond are quite stable to biotinidase. Thus, we prepared a *nido*-carborane containing biotin derivative, **20**, (Scheme 4) which contains a lysine moiety attached to the biotin for biotinidase stability.

Scheme 4: Biotinidase resistant biotin derivative containing *nido*-carborane



Animal Experiment 10 In this experiment the biotin-lysine-*nido*-carborane, **20**, was radiolabeled with I-125 and compared with another biotinidase stabilized derivative that does not contain a *nido*-carborane moiety, biotin-sarcosine-*p*-[^{131}I]iodobenzoate, **22** (Figures 10a, 10b, and Table 1). As we previously observed for *nido*-carboranes, the blood concentration is much higher for these derivatives than for biotin derivatives that only contain aryl iodides. It seems likely that the slower blood clearance observed for the *nido*-carborane containing biotin derivatives is due to binding with serum proteins. Whether the longer half-life is a problem when short-ranged alpha-

Figure 10a

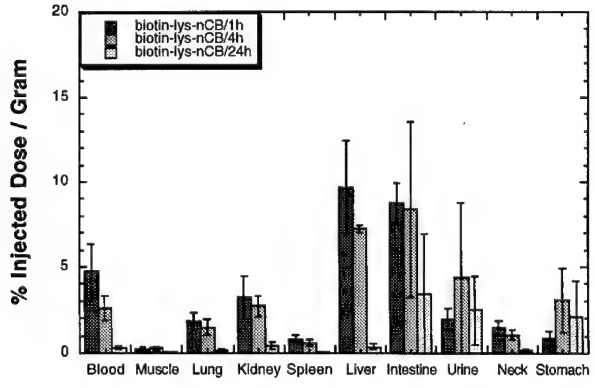
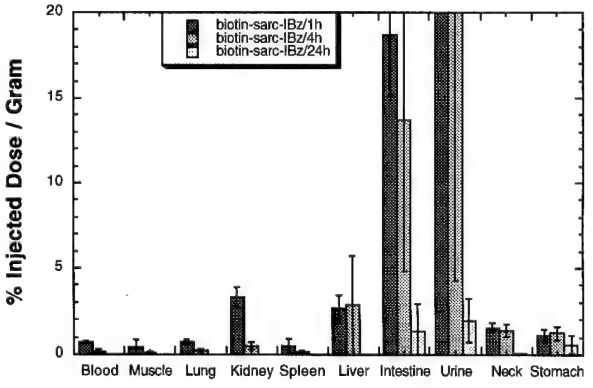


Figure 10b



emitting radionuclides are used is not know yet. The other important observation to be made about the differences in Figures 10a and 10b is that the intestine concentrations are much lower for the *nido*-carborane containing moiety. The reason for this is currently being investigated in other funded studies. The results obtained were encouraging enough to label **20** with At-211 and evaluate its in vivo properties.

Animal Experiment 11 Biotin derivative **20** was radiolabeled with At-211 and its biodistribution was compared with I-125 labeled **20** in athymic mice. The results are shown in Figures 11a and 11b. It was very encouraging to see that the At-211 on **20** appeared to be relatively stable to dehalogenase. This statement can be made because the concentrations of At-211 in the lung, spleen and stomach are low and decrease with time. Free astatide localizes in these tissues and increases with time. However, some instability may be noted as the concentration of At-211 is lower in blood than the co-injected I-125, but slightly higher in lung, kidney, and spleen at the three time points.

Figure 11a

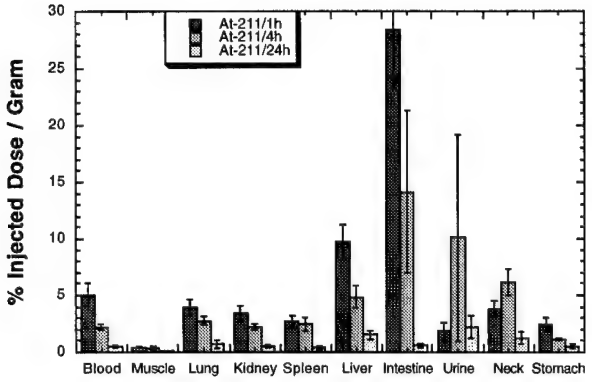
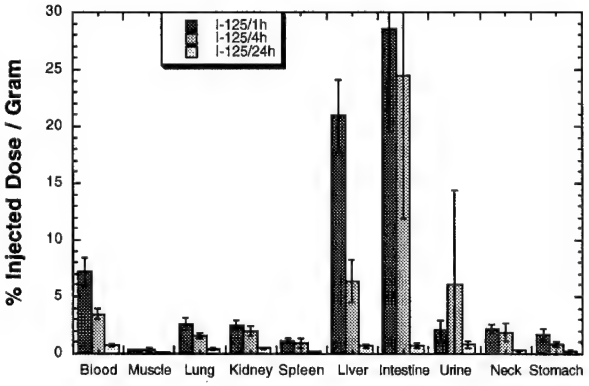
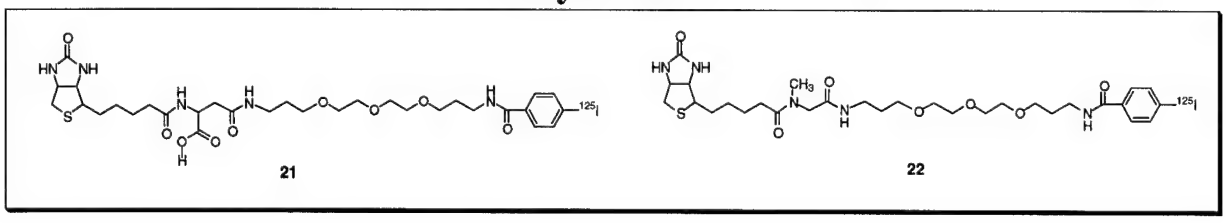


Figure 11b



The results obtained in the above experiment (#11) suggest that we have a biotin derivative which can be used for carrying At-211 to cancer cells in vivo. We were interested in determining the stability of a similar At-211 labeled aryl biotin derivative before making the final choice. The compound chosen is the biotin-aspartate-trioxatridecanediamine-iodobenzoate, **21** (Table 1). This compound was synthesized with other funding, but was available for study with At-211.

Table 1: Structures of Biotinidase Stabilized Biotin Derivatives that have Aryl Halides



Animals Experiment 12 In the twelfth animal study, we investigated the biodistribution of radioiodinated biotin derivative **21** relative to another biotinidase stabilized biotin derivative **22** (Table 1) at 1, 4 and 24h post injection. The tissue distributions of the radioiodinated biotin

derivatives are shown separately in Figures 12a (**21**) and 12b (**22**). Much more rapid blood clearance was noted relative to the *nido*-carborane derivatized biotin derivative **20**. Although the error bars were relatively large for this experiment, it was clear that biotin derivative **21** had a better distribution than the alternate, **22**. Therefore, we chose that compound to test the stability when labeled with At-211.

Figure 12a
Distribution of 21

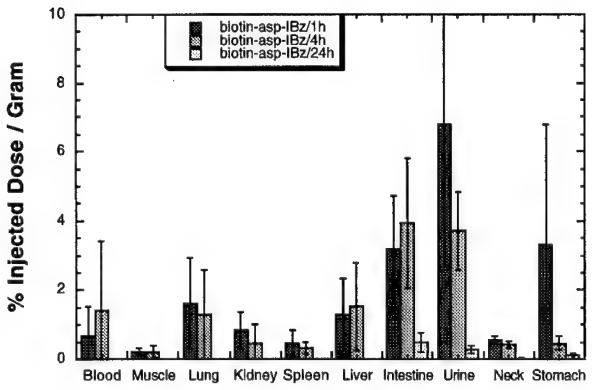
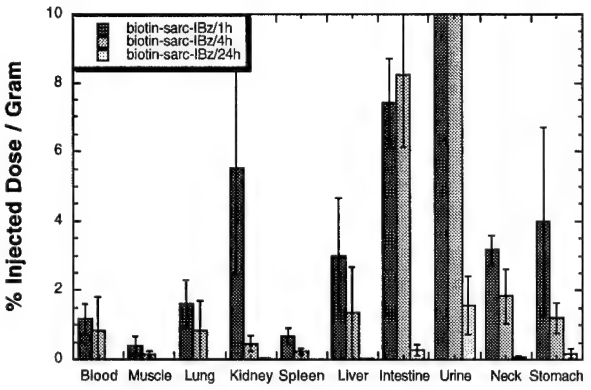
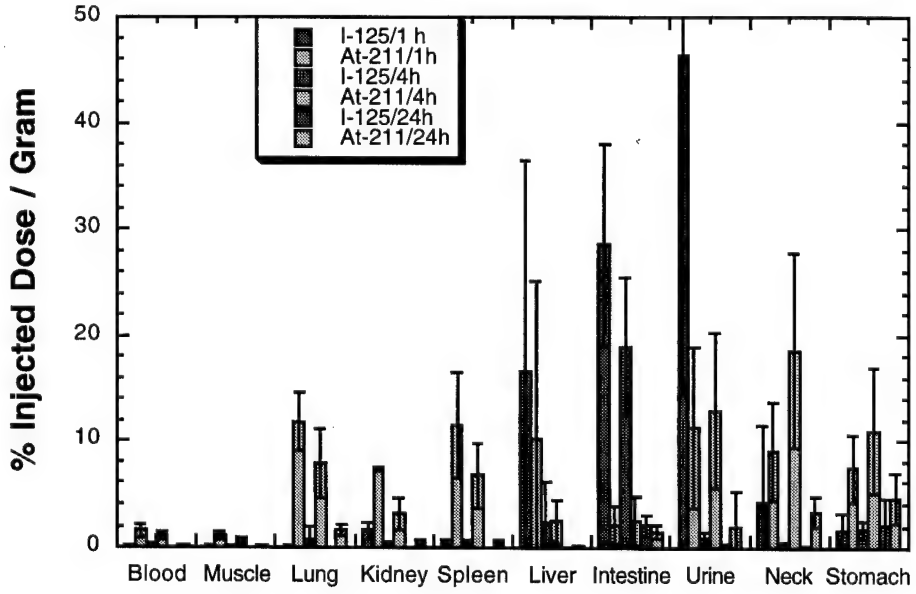


Figure 12b
Distribution of 22



Animal Experiment 13 In this experiment we evaluated the biodistribution of astatinated 21 relative to iodinated 21 at 1, 4 and 24 h post injection. The results obtained are shown in Figure 13. To indicate stability of At-211, the distributions should be the same. It is quite apparent that the distribution of At-211 and I-125 are quite different. Indeed, much higher levels of At-211

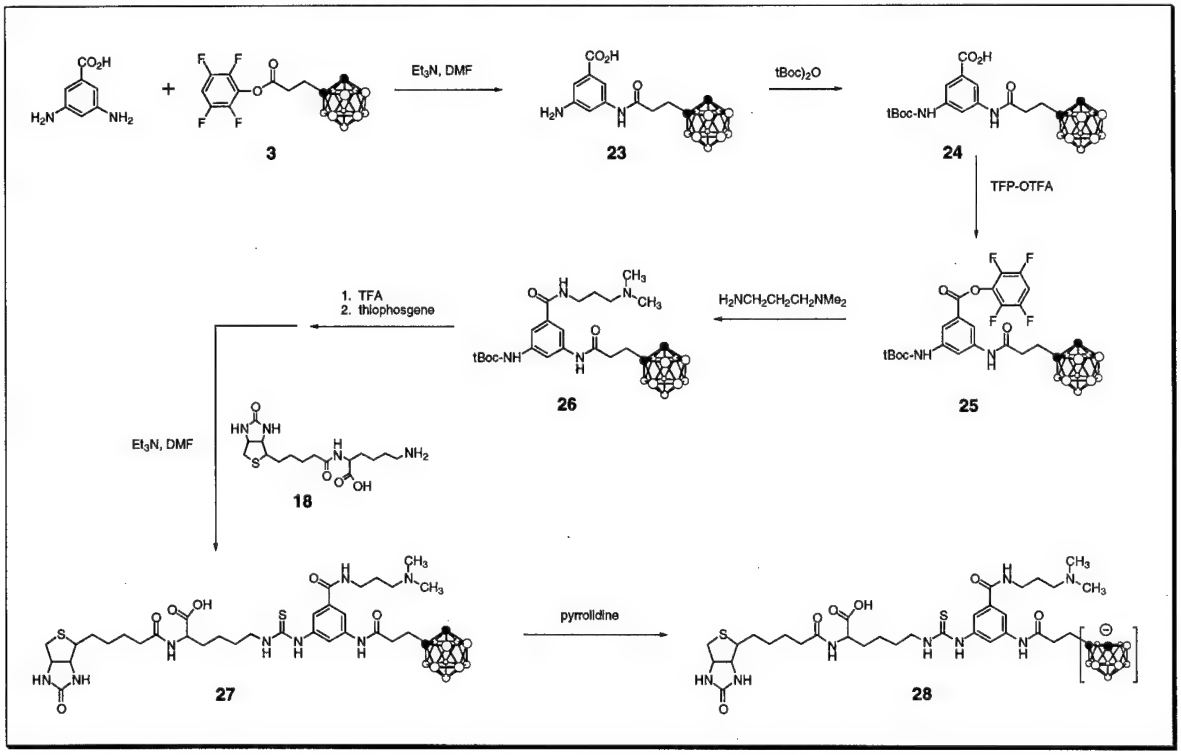
Figure 13
Biodistributions of Biotin-Aspartate-Trioxadiamine -[^{211}At]Astatobenzoate- and [^{125}I]Iodobenzoate



are seen in the lung, spleen and stomach, indicating that free At-211 is present. This result dramatically shows that the aryl group is inferior to the *nido*-carborane for carrying At-211.

Compound **20** is not an optimal *nido*-carborane configuration for biotin derivatives because of the high blood concentrations. The high blood concentrations observed for radiolabeled **20** were thought to be caused by the *nido*-carborane interacting with serum proteins. As with our other studies of *nido*-carborane derivatized SAv, we believed that incorporation of a dimethylamine functionality might provide an improved biodistribution. Therefore, we synthesized the biotin derivative **28** as depicted in Scheme 5.

Scheme 5: Biotinidase resistant biotin derivative containing *nido*-carborane that also contains a dimethylamine moiety



After synthesizing **28**, we conducted radiolabeling experiments and found that it was efficiently labeled.

Animal Experiment 14 The fourteenth animal experiment examined the biodistributions of the biotin-*nido*-carborane-dimethylamine derivative [^{131}I]**28** and the biotin-sarcosine-iodobenzoate [^{125}I]**22** in athymic mice. In the experiment, equal quantities of the biotin derivatives were co-injected and the biodistributions were examined at 1, 4, and 24h post injection. The results obtained are shown in separate graphs (14a for **28** and 14b for **22**). Unfortunately, the blood concentration did not decrease with the addition of the dimethylamine functionality (see Figure 11a). However, there appeared to be a decrease in the liver and intestine concentrations from that observed for **20**. Additional studies have been funded to continue to study the effects of alterations on the distribution of biotin derivatives.

Figure 14a
Distribution of Compound **28**

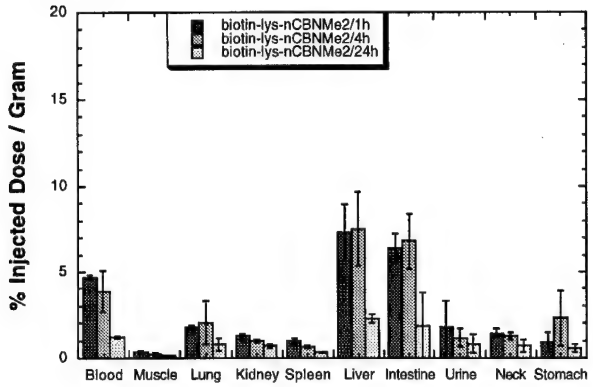
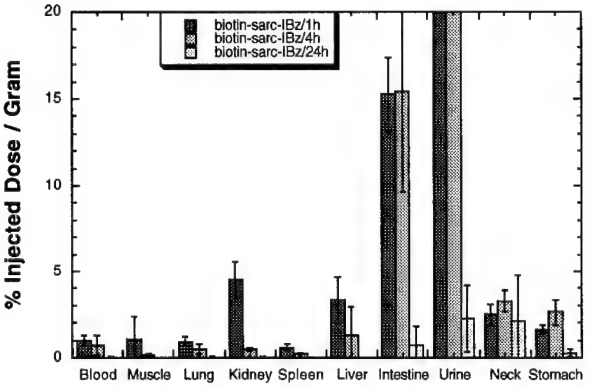


Figure 14b
Distribution of Compound **22**



Task 14: Optimize 3-step pretargeting of At-211

An animal study was conducted to evaluate tumor targeting using the 3-step approach and radioiodinated *nido*-carborane derivatized biotin,[^{131}I]**20**.

Animal Experiment 15 In the fifteenth animal study, 100 µg of biotinylated [^{125}I]107-1A4 was injected and allowed to localize to LNCaP tumor xenografts for 24h. After 24h, 100 µg of succinylated SAv was injected and allowed to circulate for another 24h. After that time, 200 µg of biotinylated and galactosylated HSA was injected to clear the biotin binding species (excess SAv and 107-1A4-SAv conjugates). Two hours after injection of the clearing agent, 1 µg of radiolabeled [^{131}I]**28** was injected. Biodistributions were obtained at 1, 4 and 24 h post injection of the radioiodinated biotin. The tissue distributions of the biotinylated [^{125}I]107-1A4 and [^{131}I]**28** are shown separately in Figures 15a and 15b (respectively). The distribution of biotin derivative was discouraging in that it did not show significant targeting to the tumor (Figure 15b). However, we have more recently learned that higher quantities of streptavidin are required to keep from getting all of the biotin binding pockets filled with endogenous biotin. Therefore, we now believe that lack of tumor targeting was not likely to happen with the quantities of reagents used.

Figure 15a

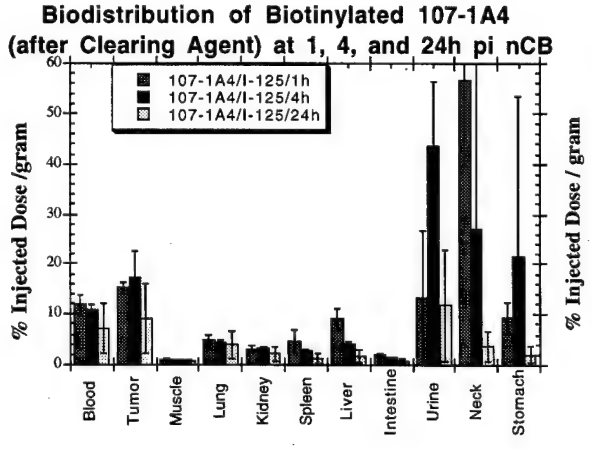
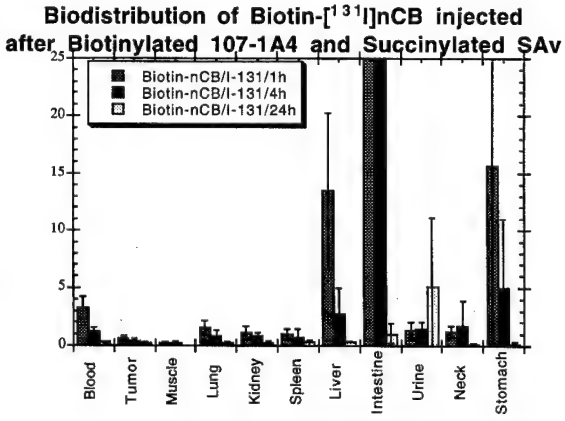


Figure 15b

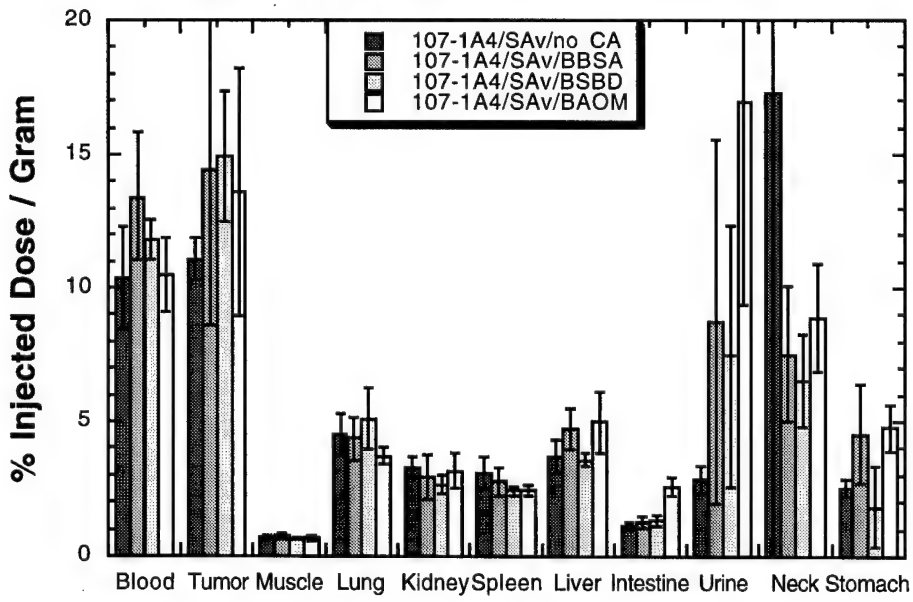


Task XX: Evaluate Alternate 2-Step pretargeting of At-211 (not part of proposed studies)

Since we were unable to optimize either the "2-step" or "3-step" approaches based on the animals study results, we decided to explore another "2-step" approach. In that approach, conjugates of the antibody and streptavidin are injected first, a clearing agent is used (step not counted), and radiolabeled biotin is administered last. We felt that this method may change the distribution with regards to the lung, liver, and spleen. An initial study was conducted to determine if a better clearing agent could be found. That study is described below.

Animal Experiment 16 In the sixteenth animal study, 150 µg of [^{125}I]107-1A4-SAv was injected into mice, after 46 h, 100 µg of either biotinylated/galactosylated BSA (BBSA) or a biotinylated/galactosylated starburst dendrimer (BSBD) or the (previously used) biotinylated/ galactosylated asialoorosomucoid protein (BAOM) was injected. Biodistributions were conducted 2h after injection of the clearing agents. The results are shown in Figure 16. As can be seen, no blood clearing was obtained. This result made us more aware of the possibility that endogenous biotin quantities may be much higher than we had previously believed. Shortly after this experiment, a publication came out that indicated 400 µg of conjugate was required to have biotin binding sites open [2].

Figure 16
107-1A4-SAv conjugate (150 ug) plus clearing agents (100 ug) at 46 h post injection



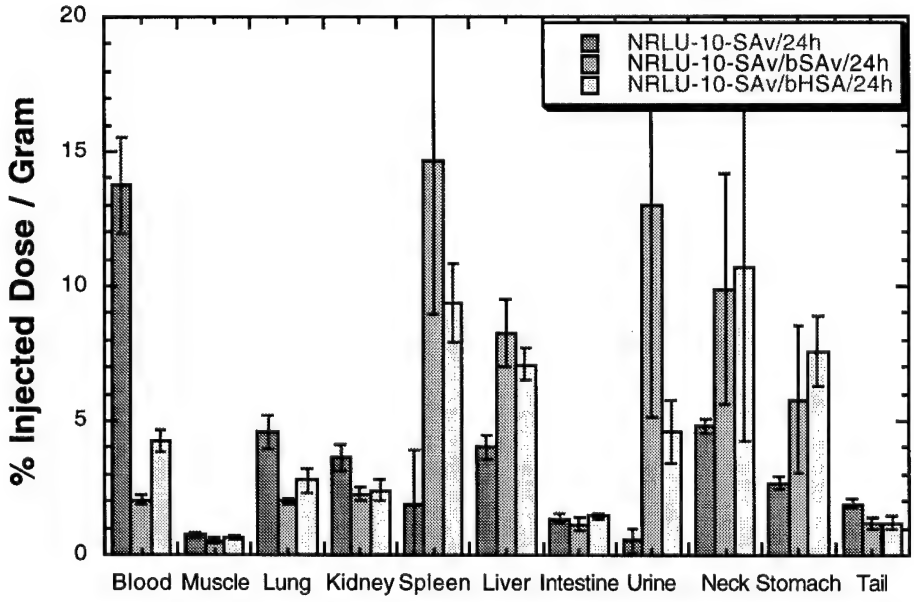
Although the experiment was not successful, it was very important as it made us acutely aware of the problem of endogenous biotin and the quantities of reagents required to off-set this problem. We were not satisfied with the clearing agents either as large quantities of biotinylated clearing agent used could result in significant increases in the serum biotin concentrations after catabolism. Therefore, we have developed a new clearing agent which is biotinidase stabilized.

That clearing agent is glycosylated streptavidin which has a biotin trimer [10] bound. We have conducted an animal experiment to test our hypothesis. That experiment is described below.

Animal Experiment 17 In the seventeenth animals study, mice were injected with 400 μg of an mAb-SAv conjugate (NRLU-10-SAv). The mAb-SAv conjugate was allowed to circulate for 23h, then 100 μg of the SAv-biotin trimer conjugate or biotinylated/galactosylated human serum albumin (HSA) was injected. In a control group, no clearing agent was administered. At 1 h after injection of the clearing agent biodistributions were conducted. The results of the tissue distribution are shown in Figure 17. Blood clearance with the SAv-biotin trimer was fairly effective, providing >86% clearance, whereas the biotinylated/galactosylated HSA provided only 71% clearance under the same conditions. Additional studies are planned to determine if 2 injections (1 h apart) of clearing agent will improve the total amount cleared from blood. Our goal is to decrease the blood level of biotin binding reagents by >95%.

Figure 17

Blood Clearance using Biotinylated and Galactosylated SAv and HSA



We have requested a no-cost extension of this grant period to conduct additional animal studies. The most important study will be to attempt to target radioiodinated biotin-*nido*-carborane, **28** to 107-1A4 pretargeted to LNCaP xenografts in athymic mice. That study has been set up and should be conducted within 2 months.

Technical Objective 4: To evaluate the localization of At-211 and I-131 in the tibia, and evaluate the effect on PSA levels.

No studies have been conducted under this objective as effective tumor targeting has not been achieved to date.

Task 15: Image LNCaP cells population in tibia with Pretargeted I-123

Task 16: Image LNCaP cells population in tibia with Pretargeted I-123

Task 17: Evaluate the Effect on Serum PSA of At-211 and I-131 pretargeted to tibia

Technical Objective 5: To assess the dose delivered to tumor xenografts and normal tissues by I-131 and At-211 in the pretargeting studies.

This objective was not done due to the lack of sufficient tumor targeting to warrant it.

Task 18: Evaluate dosimetry for optimized 2-step pretargeting

Task 19: Evaluate dosimetry for optimized 3-step pretargeting

Task 20: Re-evaluate dosimetry for optimized 2-step pretargeting for At-211 results

Task 20: Re-evaluate dosimetry for optimized 3-step pretargeting for At-211 results

Task 21: Evaluate dosimetry for pretargeting to tibia

Reports
Task 22 Annual report written and submitted
Task 23 Final report written and submitted

C. KEY RESEARCH ACCOMPLISHMENTS:

- Synthesized and conjugated streptavidin with the reagent, 1′-(3′-(isothiocyanato)anilino)-3-propionyl-*nido*-caborane and a derivative of that reagent which has a dimethylamine functionality These reagents have been shown to be useful for direct labeling of proteins with radioiodine and astatine-211.
- Synthesized two new biotin reagents which have a *nido*-carborane moiety for labeling with astatine-211 and demonstrated that they were stable to in vivo dehalogenation.
- Further characterized the anti-prostate antibody, 107-1A4, and evaluated the number of binding sites per cell in the prostate carcinoma model, LNCaP.
- Successfully biotinylated 107-1A4 and evaluated its binding and in vivo characteristics at various levels of biotinylation.
- Successfully succinylated streptavidin and evaluated its in vitro and in vivo characteristics with various levels of succinylation.
- Determined the optimum amount of avidin when used as a clearing agent in the 2-step pretargeting procedure.
- Determined the optimum amount of succinylated streptavidin to obtain tumor targeting in 2-step pretargeting procedure.
- Determined the optimal amount of the biotinylated asialoorosomucoid protein required as a clearing agent in the 3-step pretargeting procedure.
- Developed new clearing reagent made of galactosylated streptavidin and a biotin trimer. This clearing agent was shown to be more efficient than others described in the literature.

D. REPORTABLE OUTCOMES:

The following is a list of manuscripts published, manuscripts in preparation, and abstracts that have been or will be presented which were funded (some in part) by this research grant.

Papers that are published:

1. Wilbur D.S., Pathare P.M., Hamlin D.K., Stayton P.S., To R., Klumb L.A., Buhler K.R., and Vessella R.L. (1999) Development of New Biotin / Streptavidin Reagents for Pretargeting. *Biomolecular Eng. 16*, 113-118.
2. Wilbur D.S., Hamlin D.K., Pathare P.M., Buhler K.R., and Vessella R.L. (2000) Evaluation Succinylated Streptavidin as the Radionuclide Carrier in Antibody Pretargeting of Prostate Cancer. In *Isotope Production and Applications in the 21st Century*, (N.R. Stevenson, Editor), Proceedings of the Third International Conference on Isotopes, Vancouver, B.C. Sept. 6-10, 1999, pp 167-169, World Scientific Publishing Co., Inc., River Edge, NJ.

Papers in preparation that will cite funding from DOD Prostate Cancer grant.

3. Wilbur D.S., Hamlin D.K., Foulin C, Pathare P.M., Zalutsky, M., Buhler K.R., and Vessella R.L. (2001) Biotin Reagents in Antibody Pretargeting. 6. Synthesis and Preliminary Evaluation of *nido*-Carboranyl-Biotin Derivatives as Carriers of Radiohalogens. *Bioconjugate Chemistry – in preparation.*
4. Wilbur D.S., Hamlin D.K., Kegley B.B., Quinn J. and Vessella R.L. (2001) Streptavidin in Antibody Pretargeting. 4. Conjugation of *nido*-Carboranes for direct labeling with Radioiodine and Astatine-211. *Bioconjugate Chemistry* or *Nucl. Med. Biology*
5. Wilbur D.S., Hamlin D.K., Kegley B.B., Quinn J. and Vessella R.L. (2001) Biotinylated Clearing Agents in Antibody Pretargeting. Development of Clearing Agents Based on Streptavidin and Avidin binding with a Biotin Trimer. *Bioconjugate Chem. – in preparation*

Presentations where grant support was (or will be) cited.

1. Wilbur D.S., Hamlin D.K., Pathare P.M., and Kegley B.B. (1999) Synthesis of a *Nido*-Carborane Derivative for use as a Protein Conjugate for Direct Radioiodination. Presented at the 13th International Symposium on Radiopharmaceutical Chemistry held June 27-July 1, 1999, St. Louis, MO.
2. Wilbur D.S., Hamlin D.K., Pathare P.M., Chyan M.-K., and Frownfelter M.B. (1999) Optimization of Radiolabeled Biotin Derivatives for Use in Antibody Pretargeting of Cancer. Presented at the 13th International Symposium on Radiopharmaceutical Chemistry held June 27-July 1, 1999, St. Louis, MO.
3. Hamlin D.K., Wilbur D.S., Pathare P.M., Venkataraman D., Corcoran M., and Press O.W. (1999) Evaluation of Antibody Biotinylation Reagents for Application to Targeted Radiotherapy. Presented at the Third International Conference on Isotopes, held in Vancouver, BC, Sept. 6-10, 1999.
3. Wilbur D.S., Hamlin D.K., Pathare P.M., Buhler K.R. and Vessella R.L. (1999) Evaluation of Succinylated Streptavidin as the Radionuclide Carrier in Antibody Pretargeting of Prostate Cancer. Presented at the Third International Conference on Isotopes, held in Vancouver, BC, Sept. 6-10, 1999.
5. Wilbur D.S. (2000) An Overview of the Design of Biotin Reagents for In Vivo Application. (Invited) Presented at The *First International Conference on (Strept)Avidin-Biotin*

Technologies, held June 18-21, 2000 in Banff, Alberta, Canada. *Biomolecular Engineering, 16*, 159.

6. Wilbur D.S., Nguyen T.-B., Chyan M.-K., Hamlin D.K., and Kegley B.B. (2000) Radiolabeled Biotin Reagents for Application to Targeted Therapy of Cancer. Presented at The *First International Conference on (Strept)Avidin-Biotin Technologies*, held June 18-21, 2000 in Banff, Alberta, Canada. *Biomolecular Engineering, 16*, 162-163.
7. Wilbur D.S. (2000) An Overview of Astatine Chemistry and Applications (Invited) Presented at the 4th International Symposium on Radiohalogens, September 9-14, Whistler, B.C.
8. Hamlin, D.K., Kegley B.B., Wilbur D.S., Buhler K. and Vessella R.L. (2000) Radiolabeling and In Vivo Evaluation of Astatinated Succinylated Streptavidin. Presented at the 4th International Symposium on Radiohalogens, September 9-14, Whistler, B.C.
9. Wilbur D.S., Hamlin D.K., Kegley B.B., Pathare P.M., Buhler K.R., and Vessella R.L. (2000) Astatinated Biotin Derivatives for Application to Targeted Radiotherapy. Presented at the 4th International Symposium on Radiohalogens, September 9-14, Whistler, B.C.
10. Wilbur D.S., Hamlin D.K., Liang B. C.-K., Chyan M.-K., Buhler K.R., Vessella R.L. (2000) Investigation of Novel Antibody Conjugates for Use in Pretargeting of Radionuclides to Cancer. (Invited) Presented at Symposium on "Advances in Radiopharmaceutical Chemistry", Pacifichem 2000 meeting, December 14-19, Honolulu, Hawaii.
11. Wilbur D.S., Hamlin D.K., Chyan M.-K., Kegley B.B., Buhler K.R., Vessella R.L., and Brechbiel M.W. (2000) Optimization of Biotin Derivative Structure to Carry Alpha-Emitting Radionuclides in Pretargeting of Cancer. Presented at Symposium on "Radionuclides for Therapeutic Oncology" , Pacifichem 2000 meeting, December 14-19, Honolulu, Hawaii.
12. Wilbur D.S., Hamlin D.K., Quinn J. and Vessella R.L. (2001) Development of an Improved Biotinylated Clearing Agent for Pretargeting Applications. Submitted for Presentation at the 48th Annual Society of Nuclear Medicine meeting to be held in Toronto, Ontario, Canada, June 23-27, 2001.

E. CONCLUSIONS:

Although we conducted a large amount of work, we did not achieve all of the goals set out in the proposal. However, we believe that the use of astatinated compound for treatment of metastatic prostate cancer is still a realistic and worthwhile goal. The pretargeting method of delivering radionuclides to cancer cells in patients is a complex system with many variables. Due to this factor, we know that we were optimistic in our original goals. Now that we know more about the system, we believe that we are close to being able to investigate the efficacy of therapeutic protocols with it. We intend to continue to study this approach and develop reagents that will make its use more feasible. We anticipate being able to successfully pretarget At-211 to metastatic prostate cancer cells in mice in the near future.

F. REFERENCES:

[1] Wilbur, D.S., Hamlin, D.K., Vessella, R.L., Stray, J.E., Buhler, K.R., Stayton, P.S., Klumb, L.A., Pathare, P.M. and Weerawarna, S.A. (1996) Antibody fragments in tumor pretargeting. Evaluation of biotinylated Fab' colocalization with recombinant streptavidin and avidin. *Bioconjugate Chem. 7*, 689-702.

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[8] Wilbur, D.S., Stayton, P.S., To, R., Buhler, K.R., Klumb, L.A., Hamlin, D.K., Stray, J.E. and Vessella, R.L. (1998) Streptavidin in antibody pretargeting. Comparison of a recombinant streptavidin with two streptavidin mutant proteins and two commercially available streptavidin proteins. *Bioconjugate Chem. 9*, 100-107.

[9] Wilbur, D.S., Chyan, M.K., Pathare, P.M., Hamlin, D.K., Frownfelter, M.B. and Kegley, B.B. (2000) Biotin reagents for antibody pretargeting. 4. Selection Of biotin conjugates for in vivo application based on their dissociation rate from avidin and streptavidin [In Process Citation]. *Bioconjugate Chem 11*, 569-583.

[10] Wilbur, D.S., Pathare, P.M., Hamlin, D.K. and Weerawarna, S.A. (1997) Biotin Reagents for Antibody Pretargeting. 2. Synthesis and in Vitro Evaluation of Biotin Dimers and Trimers for Crosslinking of Streptavidin. *Bioconjugate Chem. 8*, 819-832.

G. APPENDICES:

All Figures, including those of reaction schemes, electrophoresis gel scans, and biodistribution graphs have been included in the Body of this report to make it easier to follow. Thus no appendices are included.

H. PERSONNEL THAT WERE PAID ON THIS GRANT

D. Scott Wilbur, Donald K. Hamlin, Reudi Risler, Brian Kegley, Ming-Kuan Chyan, Tim Nguyen, Feng Wan